DOCTORAL THESIS

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Influence of trace elements on anaerobic digestion of maize silage and a model substrate

Einfluss von Spurenelementen auf den anaeroben Abbau von Maissilage und einem Modelsubstrat

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Abstract

Anaerobic digestion of agricultural biomass and biogas production in full scale biogas plants is presently increasing and plays more and more an important role in the supply of renewable energy. Biogas plants operating exclusive of maize silage as substrate consistently show a lack of trace metals after several month of operation leading to drastic decreases in biogas production and process stability. Therefore, this study on hand is dealing with the influence of different trace elements on anaerobic digestion of maize silage and a maize model substrate.

Anaerobic batch experiments at mesophilic conditions (35°C) with maize silage as well as a defined model substrate for maize consisting of xylan (cellulose), starch, urea and posphate and addition of a selected trace element solution showed an increase of methane production by up to 30 %. The addition of nickel instead of the selected trace element solution in a concentration of about 0.6 mg kg^{-1} FM increased methane formation by similar amounts. An improvement of methane production was also demonstrated for cobalt; however, addition of molybdenum did not show a clear influence on methane production.

The limitation of nickel and cobalt in anaerobic semi-continuous fermentations with the defined maize model substrate demonstrated clearly the importance of these elements in anaerobic digestion and biogas formation. A decreased nickel dosage during the fermentation process leads to accumulation of volatile fatty acids and a concomitant drop of the pH. After gradual increase of the nickel concentration up to 0.6 mg kg^{-1} FM, the fermentation process could be operated with an organic loading rate of 4.3 g ODM L^{-1} d^{-1}. In addition, a cobalt dosage of 0.05 mg kg^{-1} FM at a nickel concentration and organic loading rate as described above did, however, enhance process stability and biogas production.

The eubacterial and archael community in a batch experiment with the defined maize model substrate and different nickel concentrations were analyzed by SSCP (single strand conformational polymorphism analysis), ARDRA (amplified rDNA restriction analysis) and fluorescent microscopy based on cofactor F_{420}. In the domain of eubacteria the major part belonged to the hydrolytic fermentative *Clostridiales* followed by *Bacilli* and *Bacteroides.* The analyzed methanogenic archea presented a clear dominance of the hydrogenotrophic *Methanoculleus* sp. which was also demonstrated by means of fluorescence microscopy.

Zusammenfassung

Die anaerobe Verwertung von landwirtschaftlicher Biomasse und Biogasproduktion in Biogasanlagen hat stark and Bedeutung gewonnen und wird eine wichtige Rolle in der zukünftigen Energieversorgung aus Erneuerbaren Energien spielen. In vielen Biogasanlagen, welche mit Maissilage als einziges Substrat betrieben werden, kann nach einigen Monaten Betriebszeit ein starkes Absinken der Spurenelemente beobachtet werden. Dieser Elementverlust führt zu einer verringerten Biogasausbeute und auch zu .einem instabilen Prozessverlauf.

In der vorliegenden Arbeit wird der Einfluss unterschiedlicher Spurenelemente auf den anaeroben Abbau von Maissilage und einem Modelsubstrat für Mais untersucht.

In anaeroben Batchversuchen unter mesophilen Bedingungen (35°C) wurden Maissilage bzw. das Modelsubstrat für Mais bestehend aus Xylan (Cellulose), Stärke, Harnstoff und Phosphor in Kombination mit einer definierten Spurenelementlösung fermentiert. Es konnten Steigerungen der Methanausbeute bis zu 30 % gemessen werden. Mit einer Dotierung von 0.6 mg kg^{-1} Frischmasse (FM) Nickel anstelle der Spurenelementlösung konnten ähnlich hohe Steigerungen der Methanmengen gemessen werden. Auch bei einer Zugabe von Kobalt anstelle der Spurenelementlösung konnten Methansteigerungen detektiert werden. Eine alleinige Dotierung mit Molybdän konnte keine eindeutige Verbesserung der Methanproduktion zeigen. In weiteren semikontinuierlichen anaeroben Fermentationen wurde der Einfluss einer Limitierung von Nickel und Kobalt untersucht. Eine zu geringe Zugabe von Nickel während der Fermentation führte zu einer Akkumulation der flüchtigen Fettsäuren und einem raschen Sinken des pH Wertes. Eine schrittweise Steigerung der Nickel Konzentrationen bis zu einem Wert von 0.6 mg kg^{-1} FM verbesserte signifikant den Fermentationsprozess. Mit dieser Nickelkonzentration konnte ebenfalls ein stabiler Fermentationsprozess nach einer Steigerung des Modelsubstrates auf eine Raumbelastung von 4.3 g ODM L^{-1} d^{-1} beobachtet werden. Dieselbe Raumbelastung konnte auch in den Kobaltversuchen bei einer Kobaltgabe von 0.05 mg kg^{-1} FM gefahren werden. Es konnte gezeigt werden, dass die beschriebenen Konzentrationen für Nickel und Kobalt bei Raumbelastungen über 4 g ODM L^{-1} d^{-1} eine Stabilisierung des Fermentationsprozesses und eine gesteigerte Biogasausbeute erzielen.

Zusätzlich wurden aus einer Bachtfermentation mit unterschiedlichen Nickelkonzentrationen und dem Modelsubstrat molekularbiologische Untersuchungen mittels SSCP (single strand conformational polymorphism analysis) und ARDRA (amplified rDNA restriction analysis) durchgeführt. Die Ergebnisse zeigten für den bakteriellen Teil eine Dominanz der hydrolytisch fermentativen *Clostridiales* gefolgt von *Bacilli* und *Bacteroides*. Bei den methanogenen Bakterien konnte eindeutig der hydrogenotrophe *Methanoculleus* sp. als dominante Spezies detektiert werden. Dies konnte auch mit Fluoreszenz mikroskopischen Aufnahmen basierend auf methanogener Eigenfluoreszenz durch den Cofaktor F_{420} bestätigt werden.

Introduction

1. Origin and History of Methanogenesis

Microbial life can be dated back to the Achaean (more than 2.5 billion years ago). These results based on the amount of biogenic isotopes differing on diversity of metabolisms and also on microfossils traces and biomarkers. The most serious biomarkers for bacterial and potential eucaryal life are given by the presence of hopanes and steranes in approximately 2.7 billion year old shale (Brocks et al. 1999; Summons 1999). The Achaean belong to a third domain of life in addition to Bacteria and Eukarya including very diverse organisms relating to metabolic activities. Archaeal microorganisms mostly assign extreme habitats with low pH, high temperature, high pressure or strong salinity (Rothschild & Mancinelli 2001). However, the Archaea include the methanogenic bacteria being responsible for methanogenesis and biogas production from organic compounds in the absence of oxygen (Barker, 1956).

Figure 1. Volta was collecting rising bubbles ("combustible air") from the marsh of Lake Maggiore in Italy - unknown artist (source: Methanogenesis - Ecology, Physiology, Biochemistry & Genetics, edited by James G. Ferry, first published in 1993 by Chapman & Hall, New York).

Back in history the scientist Earl Alessandro Giuseppe Antonio Anastasio Volta discovered “combustible air” (Fig. 1) in 1776 at the marsh of Lake Maggiore in Italy. He collected rising bubbles and already detected the formation of methane (Paoloni, 1976). In some famous letters to his friend Father Carlo Campi he wrote about experiments with this new burning gas (Ferry, 1993). Furthermore, on Nov. 5, 1783 George Washington and Thomas Paine described their experiments on the river near Rocky Hill when they disturbed the mud at the bottom of the river actually bubbles of gas rise which they can set on fire (Menzies, 1969). In the work of Paoloni (1976) he described that correspondence between Volta and important scientists of this age named this flammable air “gas hidrogenium carbonatrum” with the english terms “carbonated hydrogen” and “carburetted hydrogen”. The term “methan” or methane was resolved in 1865 and in 1892 this nomenclature was verified by an international congress on chemical nomenclature. In his book *Bacterial Fermentations* H. A. Barker recapitulate the age of famous scientists around Volta. The biological formation of methane was investigated by Volta but his theses were actually completed by William Henry in 1806 (Barker, 1956). After some decades and investigations of eminent scientists like Boussingault and Bunsen, Bechamp (1868), a student of Louis Pasteur, demonstrated at the first time that methane formation based on microbiological processes. He further described the degradation of sugar and starch added to an inorganic medium under anaerobic conditions. Bechamp named the living part in the fermentation *Microzyma cretae*. These days and for our comprehension it was a simple experiment, however Bechamp described clearly that methane can be generated by microorganisms from substrates such as ethyl alcohol and calcium carbonate. Continuative experiments accomplished by Tappeiner with plant material, three identical anaerobic cultures and contents of the ruminants intestinal showed that only untreated cultures produce methane (Tappeiner, 1882). Further investigations in the last quarter of the 19th century were realized by using cellulose as fermentation substrate. In fact cellulose is the major fraction in plants it was not devious to suppose that this polymer is a primary source for methane formation (Barker, 1956). In several experiments the scientists Popoff, Tappeiner and Hoppe-Seyler were dealing with cellulose and cultures of bacteria from soil or the intestinal tract of herbivorous animals. They reported that degradation of cellulose under anaerobic conditions produced different intermediates like carbon dioxide,

acetic and butyric acid and also hydrogen (Popoff, 1875, Hoppe-Seyler, 1886). Nevertheless, they wrongly concluded that the bacteria which produced the intermediates also form the generated methane. Omelianski (1906) was one of the investigators who tried to explain that the decomposition of cellulose to intermediates and following methane formation occurred by a two-stage process. However, he could not describe the methane formation process in detail but he illustrated the involvement of methane producing microorganisms, in short the methanogenic archaea.

Stephenson and Stickland (1933) introduced the modern period of methane research. In a study they demonstrated the isolation of a microorganism which oxidized hydrogen and reduced one-carbon compounds like formate, carbon dioxide, etc. to methane. H. A. Barker, the most important scientist in recent studies of methanogenesis obtained in his postdoctoral era *Methanobacillus omelianskii* as well as *Clostridium kluyveri*. His works actually impress the following 30 years since 1936 relevant the investigations in methanogenesis and biogas formation. He further developed a standard technique to determine colonies of methanogens (Barker, 1940). Schnellen (1947) discovered and explored in the laboratory of Kluyver in Delft the pure cultures *Methanobacterium formicicum* and *Methanosarcina barkeri.* In further studies he reported that the methanogenic bacteria *Methanosarcina* converted methanol to methane in a ratio of 4 moles methanol to 3 moles methane and 1 mole carbon dioxide. At the same time Buswell and Sollo (1949) adapted the general concept that the methanogenic bacteria reduce carbon dioxide to produce methane. They added ^{14}C-labeled carbon dioxide to acetate-fermenting cultures and observed that only traces of labeled carbon were found in the methane. Stadtman and Barker (1949) corroborated the results of Buswell and Sollo. Due to the fullness of investigations they postulated two paths of methane formation. The first parth occurred by the reduction of carbon dioxide and the second by reduction of a methyl group which led to a common precursor of methane for each path. However, since the 1950s an opulence of reported studies leads to a better understanding of anaerobic decomposition of biomass and methane formation. Moreover, this decade was the beginning of relevant investigations of methyl group conversion to methane (Ferry, 1993). Pine and Barker (1956) labelled in their experiments the three hydrogen atoms of the methyl carbon of acetate with deuterium to observe a feasible formation of methane during fermentation. In further experiments with deuterium

labeld water and the described hydrogen labelling of the acetate methyl group they were able to demonstrate that during methyl group reduction one proton came from the water molecule and the methyl group was transferred complete to the end product methane. These results had an important influence on understanding of the terminal step of methane formation and the reducing of carbon dioxide to methane. The isolation and characterization of *Methanobacterium ruminantium* by Smith and Hungate was (1958) another essential step for a continuative comprehension of anaerobic digestion and methane formation. The following exemplary extract could only display a small overview and should, however, summarize historical evidence of methanogenesis.

The today established Hungate technique was developed and standardized for the cultivation of strictly anaerobically growing microorganisms (Hungate, 1969). Johns and Barker (1960) started the era of cell physiological studies and investigations of the enzymology of methanogenesis at the beginning of the 1960s with a study about the influence of hydrogen on the culture of *M. omelianskii*. The important results of Blaylock and Stadtmann (1963) for a better understanding of the enzymology of methyl group reduction to methane are based on previous studies of Guest et al. (1962). They showed that methylcobalamin could supply the methyl group for a reduction to methane. From previous studies it was known that methanogenic bacteria stored high amounts of vitamin B_{12}, nevertheless Lezius and Barker (1956) reported that the corrinoid of *M. omelianskii* was characterized as a 5-hydroxy-benzimidazolecobamide derrivate called Factor III. The intensive research on methylcobalamin supported the discovery of coenzyme M in the year 1971 by McBride and Wolfe. The importance of ferredoxin in acetaldehyde oxidation was observed in cell extracts of *M. omelianskii* (Brill and Wolfe, 1966). The application of electron microscopy on determination of hydrogen-grown methanogenic bacteria was reported in 1968 (Langenberg et al., 1968). The overall examined single culture of *M. omelianskii* was intensely investigated by Bryan et al. (1967) and determined as an association of two microorganisms in 1967. Cheeseman et al. (1972) isolated and purified a new fluorescent compound from the *Methanobacterium* strain M.o.H. the so called cofactor F_{420}, with an absorption peak at 420 nm in the oxidized form. The structure of coenzyme M an important enzyme in methanogenesis was first characterized by Taylor and Wolfe (1974). In the year 1979 Balch and Wolfe reported that coenzyme M was only found in methanogenic bacteria. Some scientists around

Whitman reported in 1980 that the cofactor F_{430}, the prosthetic group of the enzyme methyl-coenzyme M-reductase includes a central nickel atom at a tetrapyrrole (Whitman and Wolfe, 1980; Diekert et al., 1980). 1982 Romesser and Wolfe discovered a new factor for carbon dioxide reduction (CDR-factor) to methane. In the middle of the 1980s the era of cloning and expression of genes of methanogenes started. For instance Alex et al. (1990) reported on a experiment where they cloned and expressed several genes which involved the F_{420}-reducing hydrogenase from a *M. thermoautotrophicum* strain. Furthermore, Peinemann et al. (1990) reported that the ATP synthesis is coupled to the reduction of CoM-S-S-HTP via electron transfer from H_2. Via NMR-spectroscopy Lin and Jaun detected (1991) a methyl-nickel (II) derivative of the coenzyme F_{430} in 1991. However, during these decades of methane science it was already investigated that nearly 70 % of the generated methane was produced from acetate via acetotrophic methanogens. There are now some discussions that the methane produced during the decomposition of plant biomass is mainly generated by hydrogenotrophic methanogenes from the orders *Methanomicrobiales* and *Methanobacteriales* (Scherer et al., 2009).

2. Microbiology and Biochemistry

Anaerobic degradation of biomass and methane formation is a multi-step process including a consortium of syntrophic microorganisms. In a first step hydrolytic active bacteria like cellulolytic *Clostridia* and *Bacilli* degrade polysaccharides and heteropolysaccharides such as cellulose and xylan into mono-, di- and oligosaccharides (Bayer et al., 2004; Cirne et al., 2007; Lynd et al., 2002).

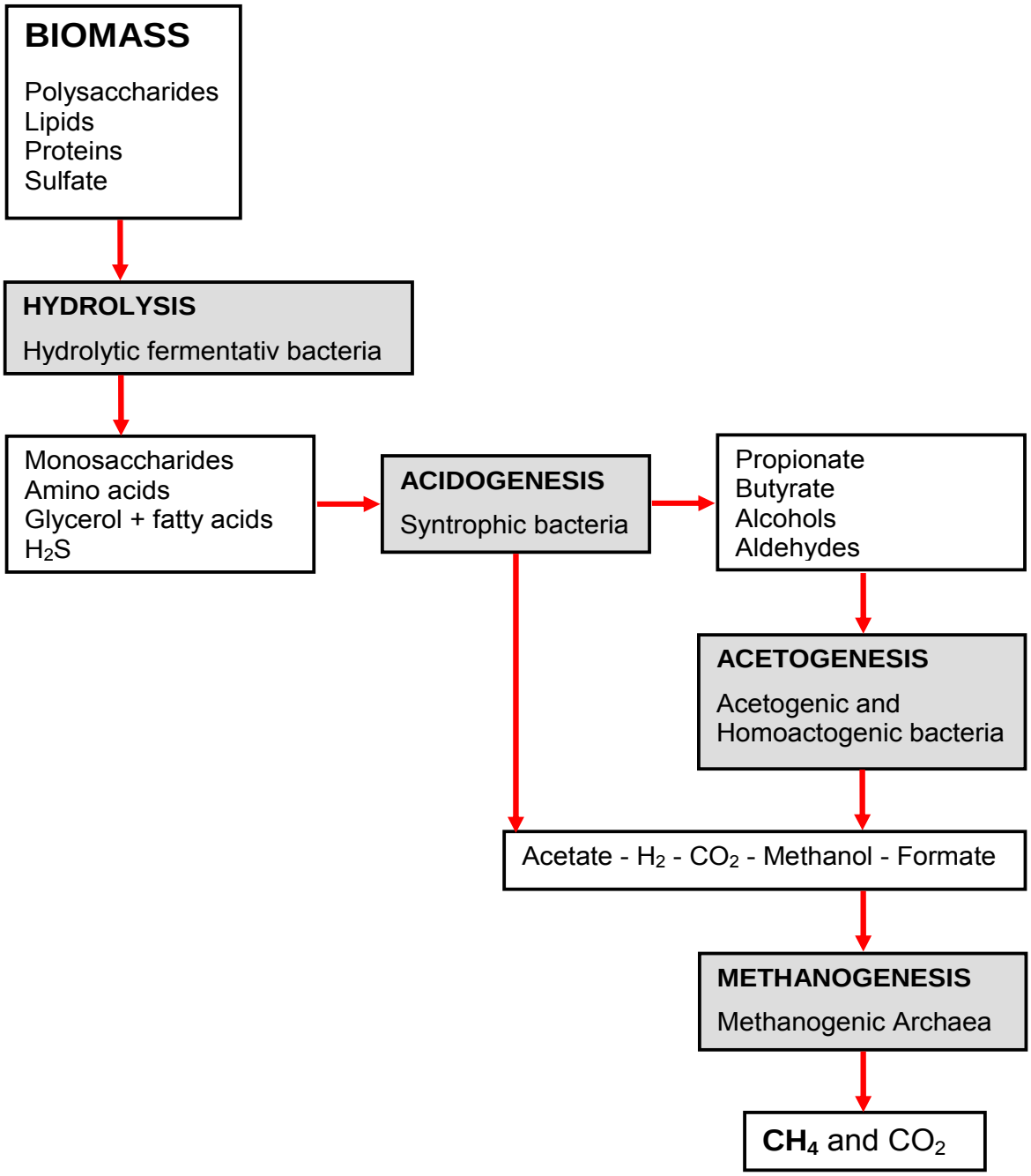

Figure 2. Schematic illustration of the important steps in methane formation. The acidogenesis and acetogenesis occur simultaneously, intermediates are substrates for following methanogenesis.

However, hydrolysis and the following acidogenesis and acetogenesis are driven by members of the domain Eubacteria. Intermediates from hydrolysis (monosaccharides, amino acids, gycerol, etc.) were converted to organic acids, alcohols and aldehydes during acidogenesis (Fig. 2). Due to initial hydrolysis and acidogenesis in the acetogenesis step more or less parallel acetogenic and homoacetogenic bacteria produce acetate, CO_2, H_2, methanol and also formate (Drake et al., 1997, 2002; Myint et al., 2007; Shin and Youn, 2005). The final products methane and CO_2 are generated by archaeal methanogenic bacteria in the methanogenesis step (Deppenmeier et al., 1996).

Cellulose Degradation - Hydrolytic Fermentativ Bacteria

Cellulose is the major component of plant cell walls and is found in an average of 35 to 50 % of plant dry weight with main differences in structure and composition depending on the plant type (Lynd et al., 1999). Furthermore, the cellulose is not a single compound and mostly present in a matrix with other biopolymers like hemicellulose and lignin (Marchessault and Sundararajan, 1993). A further extraordinary detail is the crystalline structure of cellulose which makes this polymer unique compared to other polysaccharides. The cellulose is naturally synthesized as single molecules which start a self-assembling cascade during biosynthesis (Brown and Saxena, 2000). Lynd et al., (2002) described in a review that purified cellulose which are used for the investigations of cellulose hydrolysis are strongly diversify in structure and availability for involved microorganisms and the choice of the substrate greatly affect the results.

As described the hydrolysis and utilization of cellulose were carried out by a diversity of microorganisms. Nevertheless, to specify the taxonomy of cellulolytic species the most abundant studies based on the characterization of small subunit rRNAs, for instance the 16S rRNA of procaryotes and 18S rRNA of eucaryotic organisms, respectively (Olsen et al., 1994; Woese, 2000). Results based on phylogenetic trees constructed from arrangment of sequenced PCR products displayed a high number of cellulose degrading organisms in the domain of *Bacteria*, several representatives within the domain *Eucarya*, especially in the fungal groups and no members of the

domain *Archaea*. Apart from members of the eucaryal fungal group there are aerobic and particularly interesting anaerobic representatives within the eubacteria.

With respect to fermentative anaerobes, the typically gram positive members are located at the genera *Clostridium, Ruminococcus, Caldicellulosiruptor*, further*, Spirochaeta, Eubacterium* and related to *Clostridium, Butyrivibrio* and the gram negative *Acetivibrio* and also important the gram negative *Fibrobacter* (Lynd et al., 2002). However, members of the genus *Cellulomonas* are the only described facultatively anaerobic cellulolytic bacteria (Bagnara et al., 1987).

In general three prominent types of enzymatic activities can be described: exoglucanases, cellobiohydrolases and endoglucanases, which are able to cleave randomly within the cellulose chain and generate new chain ends and different oligosaccharides. Exoglucanases can also process microcrystalline cellulose (Henrissat et al., 1998; Teeri, 1997). However, anaerobic cellulolytic bacteria degrade cellulose mainly by assistance of different complex cellulase systems well described for *Clostridium thermocellum* (Schwarz, 2001). Schwarz further reported that the majority of anaerobic cellulolytic bacteria have located their cellulases on the cell surface and degrade cellulose when directly attached to the substrate (Fig. 3).

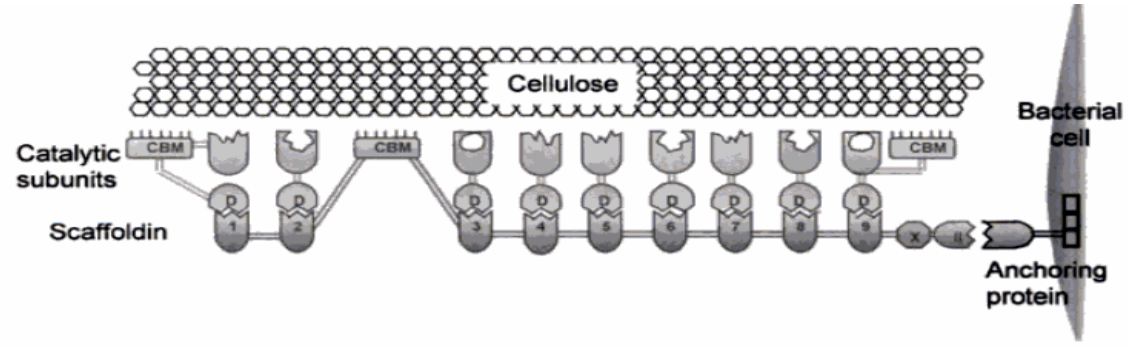

Figure 3. "Schematic illustration of the *Clostridium thermocellum* cellulosome and an associated cell-surface anchoring protein" (source: Handbook on Clostridia, edited by Peter Dürre, published in 2005 by CRC Press, Taylor and Francis Group, 6000 Broken Sound Parkway NW, Boca Raton, FL 33487-2742).

The anaerobic cellulolytic bacteria absence the ability to an effective cellulose degradation. As an alternative path they produce a complex cellulase system, the cellulosome (Fig. 3). The cellulosome enables an optimal "reaction chamber" for the cellulase-substrate contact. Cellulosomes are stable enzyme complexes produced

and established on the cell wall in presence of cellulose and can also bind to microcrystalline cellulose. The cellulosome structure of different anaerobic bacteria from *Clostridium* sp*.* and *Ruminoccus* sp. are similar and well studied (Schwarz, 2001; Bayer et al., 1994). However, most cellulases and also the cellulosome offer a modular structure including catalytic and carbohydrate-binding modules (CBMs). The CBM (Fig. 3) binds to the almost insoluble cellulose surface and enhances cellulose hydrolysis by convergency of the substrate and the catalytic domain (Din et al., 1994). For instance as described in the review of Lynd et al. (2002) a high rate of degradation of microcrystalline cellulose was observed in a preparation of a *C. thermocellum* cellulosome. Amongst others, the high ratio of substrate conversion could be attributed to the presence of interacting cellulolytic and hemicellulolytic enzymes in the cellulosome. Via electron microscopy Bayer et al. (1998) demonstrated a compact “fist”- like structure of the cellulosome which changes its conformation when attaching to the microcrystalline cellulose for a temporary docking of the catalytic domain. Above all, the cell wall and the cellulosome constitute a contact region which avoids diffusion of the substrate oligosaccharides into the enclosed environment.

Energetics and Syntrophic Cooperation with Methanogenic Bacteria

Methanogenesis is the final electron-accepting process in anaerobic environments such as swamps, paddy fields, ruminant intestinal tract, and also during anaerobic fermentation of biomass in biogas plants and sewage treatment plants. However, anaerobic degradation of biomass, for instance hexose to methane and carbon dioxide gained approximately 15 % of energy compared to aerobic degradation (Fig. 4). Due to this fact methanogenesis will be the last step after all other electron acceptors have been reduced. Moreover, the end product methane stores a lot of energy which can be utilized in oxygen dependent processes, e. g. as substrate for methane oxidizing bacteria (Schink, 1997).

a) $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$ ($\Delta G°' = -2.870$ kJ · mol^{-1})

b) $C_6H_{12}O_6 \rightarrow 3\,CO_2 + 3CH_4$ (($\Delta G°' = -390$ kJ · mol^{-1})

Figure 4. General schedule of energy recovery of aerobic (a) and anaerobic (b) degradation of organic matter.

Syntrophically operating anaerobic microorganisms are experts in energy utilization. Bacteria cooperating in synthropic anaerobic fermentations can grow with a minimum of about -20 kJ mol^{-1} ATP to obtain free energy for further reactions (Schink, 1990). The cooperation of anaerobic methane and carbon dioxide producing communities depends on different groups of bacteria including primary and secondary hydrolytic fermenting bacteria, as well as homo- and heteroacetogenics (Fig. 2) and two types of methanogenic archaea, the acetoclastic and hydrogenotrophic methanogens (Bryant, 1979).

The anaerobic consortium in the methane formation process utilizes the advantage of a close contact and aggregation between symbiotic bacteria and methanogens for an optimal metabolite transfer (Schink and Thauer, 1988). However, under special conditions such as sulfate-rich environments the primary fermenting bacteria benefit from substrate conversion of hydrogen-oxidizing bacteria. Moreover, a relatively low hydrogen partial pressure (<10 Pa) offer electrons at a redox potential of NADH (-320mV) to release molecular hydrogen. Due to this important event, fermentation conditions can change to the production of more CO_2, acetate and hydrogen instead of butyrate and ethanol formation, leading to an increase in ATP synthesis (Thauer et al., 1997). The balance of hydrogen production in anaerobic fermentation of biomass plays an important role for a stable fermentation process. If hydrogen production increases because of inhibition of hydrogenotrophic methanogens a consequent drop of pH (<6) and a accumulation of volatile fatty acids lead to instability of the fermentation process or in the last resort to a stop of the fermentation (Zeikus, 1977; Zehnder and Brock 1979).

Anyway, in syntrophic community interacting bacteria groups which operate close-partnered can oxidize hydrogen as well as formate in the same electron transfer process (McInerney et al., 1979). The redox potential of CO_2/formate is similar to H^+/H_2 with -420 and -414 mV respectively. Bacteria which are involved in nearly identical interspecies electron transfer exchange formate with hydrogen and also

backwards (Bleicher and Winter, 1994). Basically there is no difference in energetics between the hydrogen and formate carrier system. The measurement of formate in low concentrations is still a problem. Actually there is no method to measure formate concentration less than 10 – 1 µM while the hydrogen partial pressure can be measured down to 10^{-2} Pa. As an alternative technique accumulated formate is determined via adoption of inhibitors (Schink, 1997; Schink, 1994).

A further important pathway in methane formation is the oxidation of acetate. Syntrophic fermentative bacteria produce beside hydrogen and formate also acetate. In the literature two major mechanisms are described. First of all the direct conversion of acetate to methane and carbon dioxide ($CH3COO^- + H^+ \rightarrow CH_4 + CO_2$) by acetoclastic methanogens and the second one as a two-step reaction with H_2 and CO_2 as intermediates which were immediately converted to methane. The second pathway is carried out by acetate-oxidizing bacteria in combination with hydrogenotrophic methanogenic bacteria (Karakashev et al., 2006).

However, homoacetogenic hydrogen oxidation generates less energy compared to hydrogen oxidation. Hence under standard conditions hydrogen oxidation is preferred by methanogenic bacteria (Dimroth, 1983; Thauer et al., 1977). Moreover, temperature is an important factor for the change in the methanogenesis pathway from hydrogen oxidation to acetate oxidation by homoacetogens. For example under psychrophilic conditions in paddy fields and lake sediments an increase of homoacetogens can be observed (Conrad et al., 1989). Furthermore, under special conditions (H_2 partial pressure <10 Pa, 10µM acetate) methane formation from acetate occurs at 5°C gaining the same energy as hydrogen oxidation at 35°C (Zehnder et al., 1982). Nonetheless, the exact function of homoacetogenic bacteria is not well described and understood yet (Schink, 1994).

The oxidation of propionate is also a relevant step in decomposition of biomass to methane. This decomposition pathway needs the syntrophic association of acetogenic and methanogenic bacteria. The methanogens metabolize the intermediates formate and H_2 immediately and thus enable propionate oxidizing by syntrophic propionate oxidizers. Members of *Syntrophobacter* clustered with *Deltaproteobacteria* can also utilize sulphate as an electron acceptor for propionate oxidizing (Frank et al., 2005). Furthermore oxidation of propionate is basically the process reverse to the fermentative propionate formation with intermediates such as methyl-malonyl-CoA, succinate, malate, pyruvate and acetyl-CoA. The CoA transfer

from acetyl-CoA or succinyl-CoA is in generally the substrate activation step (Houwen et al., 1990). However in a study of de Bok et al. (2001) with a coculture of *S. propionica* and *M. hungatei*, the obtained results indicate that the syntrophic *S. propionica* does not oxidize propionate using the methyl-malonyl-CoA or the reductive carboxylation way. They further propose an alternative propionate degradation pathway via disproportionation to actetate and butyrate via a six-carbon intermediate.

Nevertheless, this chapter can only be a short overview of biochemistry and syntrophic cooperation in methanogenesis. We did not discuss the butyrate, glycolate and ethanol oxidation; further the oxidation of aromatic compounds and branched-chain fatty acids and many other components to complete the understanding of metabolism in the methane formation process.

Enzymes and Co-Factors in Methanogenic Archaea

The major and most phylogenetically various group in the Archaea domain, the so called methanoarcheaea, developed evolutionary different energy-yielding pathways which are characterized by one-carbon biochemistry supporting novel enzymes and cofactors. Moreover, these pathways corporately enforce the two-electron reduction of methyl-coenzyme M to methane catalyzed by methyl-coenzyme M reductase but differ in the used source of the methyl group transferred to coenzyme M. Acetate is the main substrate in biomethanation. However, using this pathway the activated substrate is cleaved by the CO dehydrogenase/acetyl-CoA synthase complex and the methyl group is transferred to coenzyme M by methyltetrahydromethanopterin or methyltetrahydrosarcinapterin. Furthermore, in the second pathway the intermediates formate or H_2 are oxidized and the gained electrons further reduces carbon dioxide to the methyl level and provides also reduction of methyl-coenzyme to methane. Additionally methane is also generated from the methyl groups of methanol and methylamines. In both pathways special methyltransferases transfer the methyl groups to coenzyme M. A change in the carbon dioxide pathway leads to an oxidation of the methyl groups to carbon dioxide. The gained electrons from this reversal step are further used for reduction of the methyl-coenzyme M. However, the

understanding of the enzymology of one-carbon reactions actually increases with respect to the physiology and molecular biology of methane formation (Ferry, 1999).

In the following description of several enzymes and cofactors the importance of heavy metals such as nickel, cobalt, molybdenum, tungsten, manganese, selenium, iron, zinc, or copper can be recognized (also see Table 1 and Fig. 5). Therefore we will describe the methyltransferase, the CO-dehydrogenase/acetyl-CoA synthase complex and the methyl-CoM-reductase, the key enzyme in methane formation.

Table 1

Enzymes and microorganisms involved in methane formation process[a]

Enzyme	Organism(s)	Metal	Reference
Methyltransferase	Methanogens and acetogens	Co(B_{12})	Beveridge and Doyle, 1989
CO-dehydrogenase	Methanogens and acetogens	Co, Ni, Fe	Ferry, 1999
Acetyl-CoA synthase	*Moorella thermoacetica*	Fe, Ni, Cu	Seravalli et al., 2003
Methyl-CoM-reductase	Methanogens	Ni	Hausinger, 1994
Hydrogenase	*Desulfovibrio*	Ni, Fe, Se	Fauque et al., 1988; Albracht, 1994
MMO[1] (free)	*Methylosinus trichosporium*	Fe	Lipscomb, 1994
Formiate dehydrogenase	*Methylobacterium*	Mo or W	Girio, et al., 1992
Formylmethanofuran-dehydrogenase	*Methanobacterium thermoautotrophicum*	Mo or W[2]	Bertram and Thauer, 1994
Aldehyde-oxydoreductase	*Clostridium*	Mo or W	White and Simon, 1992
Nitrogenase	*Methanosarcina barkeri*	Mo or V, Fe	Chien et al., 2000

[1]Methane monooxygenase

[2]An iso-enzyme was synthesized when tungsten was added to the fermentation medium.

[a]Table is modified from the reference **Zandvoort et al., 2006**

The methyltransferase is an integral membrane-bound complex and generates additionally to methyl transfer a sodium ion gradient across the membrane (Becher et al., 1992). The enzyme incorporated a corrinoid cofactor (5´-hydroxybenzimidazolyl cobamide, see also Fig. 5) and catalyze two partial reactions (Gartner et al., 1993). In the first reaction the Co^{1+} acts as a super-reduced nucleophile which accepts the methyl group from CH_3-H_4MPT. In the second reaction a transfer of the methyl group from CH_3-Co^{3+} to coenzyme M (Fig. 5, HS-CoM) is carried out with a following generation of CH_3-S-CoM and regeneration of the activated corrinoid form, Co^{1+}. Moreover, the transfer of methyl depends on functions including sodium and a pumping process of this cation through the membrane (Gartner et. a., 1994; Weiss et al., 1994).

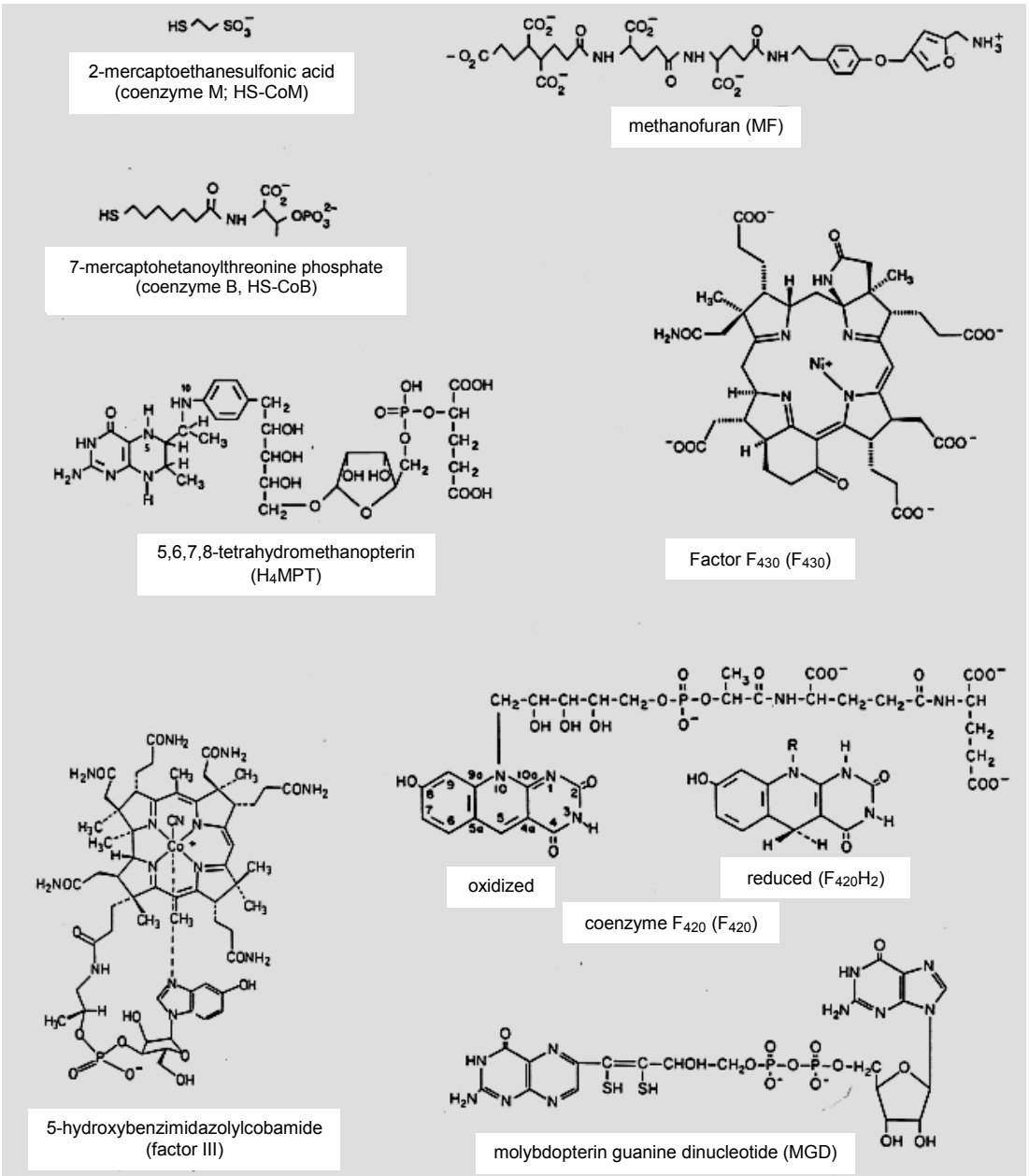

Figure 5. “Structures of cofactors required for catalysis of one-carbon reactions in methanogenic pathways”. Figure modified from reference **J. G. Ferry, 1999.**

The methyl-coenzyme M reductase from *M. thermoautotrophicum* is the best investigated reductase in methanogenesis. The catalyzed reaction actually occurs in all methanogenic pathways. The utilized electron donor for all reductases is coenzyme B (CoB) illustrated in Fig. 5. The heterodisulfide CoM-S-S-CoB is another product beside methane (Ferry, 1999). However, two genetically different isoenzymes of *M. thermoautotrophicum* are desribed by Rospert et al. (1990) (MCR I and MCR II) which are containing in each case two molecules of the yellow nickel-including porphinoid cofactor F_{430} (Fig. 5). The regulation of the expression of MCR I or MCR II respectively depends on the concentration of H_2 in the fermentation medium which correlates with the distinct expression of the F_{420-} and H_2-dependent dehydrogenases (Morgan et al., 1997).

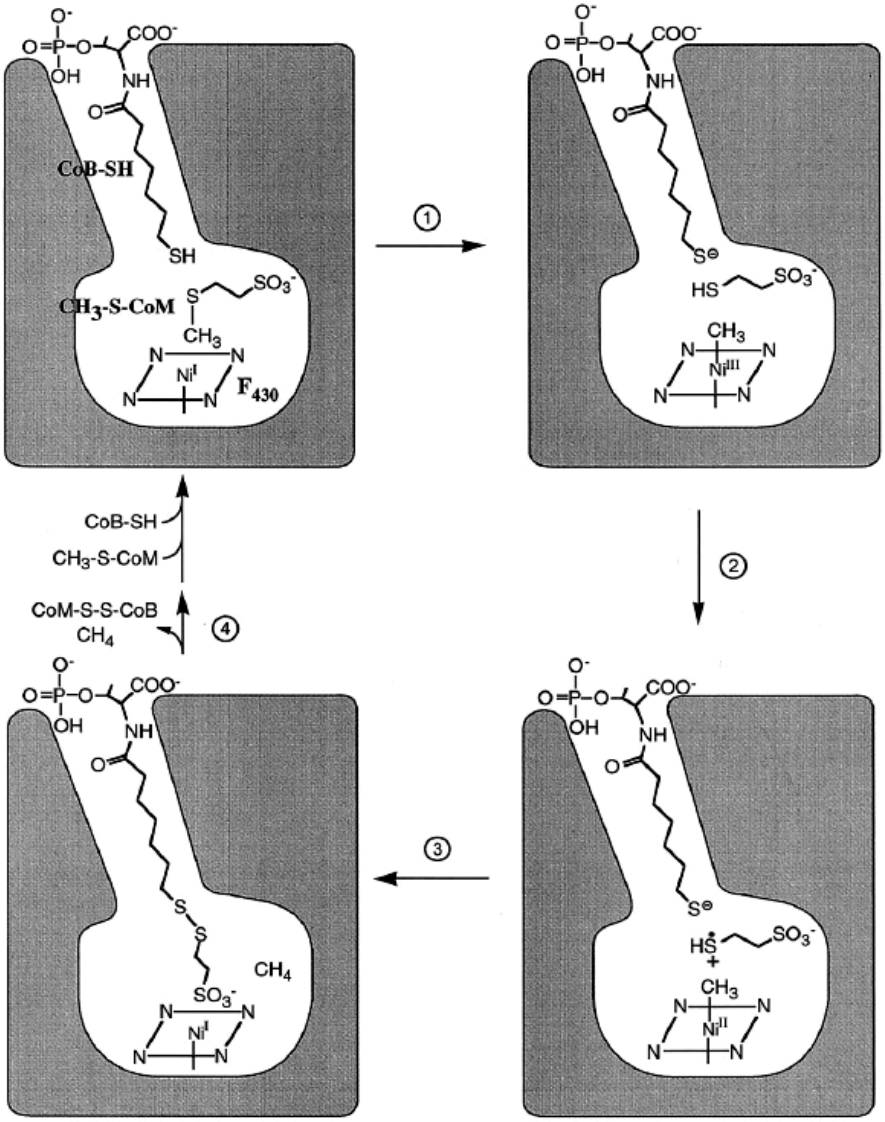

Figure 6. Proposed steps in the mechanism of methyl-CoM reductase. Figure modified from reference **J. G. Ferry, 1999.**

Moreover, F_{390} a degradation product of F_{420} generated under oxidative stress, play a role in regulation of methylreductase and the F_{420}- and H_2-dependent dehydrogenases (Pennings, et al., 1998). Based on the crystal structure of methyl-CoM reductase four different reaction steps suggested (Fig. 6). In the first step a nucleophilic attack of Ni(I) on CH_3-S-CoM and generation of a [F_{430}]Ni(III)-CH_3 intermediate occurs while CoM, CoB and F_{430} hold together their relative positions. In the second step through oxidizing of HS-CoM by Ni(III) a ˙S-CoM thiyl radical and [F_{430}]Ni(II)-CH_3 intermediates are produced. In the third step protonolysis releases CH_4 and CoB-S-S-CoM is also produced by coupling of the thyil radical to ˉS-CoB.

Furthermore the excess electron which is transferred to Ni(II) reduce it to Ni(I) (Goubeaud et al., 1997; Becker and Ragsdale 1998).

The CO-dehydrogenase/acetyl-CoA synthase complex is an important enzyme in the acetate conversion to methane. The enzyme complex includes five subunits and cleaves the C-C and C-S bonds in the acetyl moiety of acetyl-CoA. It further oxidizes the carbonyl group to CO_2 with the activity of the CO dehydrogenase and transfers in a final step the methyl group to Tetrahydrosarcinapterin (H_4SPT) an analog to the H_4MPT described in Fig. 5 (van Beelen et al., 1984). However, the synthase complex contains a nickel/iron-sulfur (Ni/Fe-S) and a corrinoid/iron-sulfur (Co/Fe-S) component and an uncharacterized third component (Abbanat and Ferry, 1991). There are three metal clusters located in the Ni/Fe-S component. Cluster A was characterized to cleave or synthesize the C-C and C-S bonds of acetyl-CoA. Furthermore, cluster A is proposed to be a novel Ni-X-[Fe_4S_4] metal center wherein X is described as an unidentified bridging atom (Lu et al. 1994; Ragsdale, 1998). Cluster B incorporate a common Fe_4S_4 center acting as an electron shuttle for cluster C. The third metal cluster (C) is also characterized as a novel bimetallic Ni-X-[Fe_4S_4] cluster and is specified as the site for CO dehydrogenase activity (Hu et al., 1996).

3. Biogas Production and Energy Recovery

The production of biogas and utilization of the energy rich gas methane for power, heat and fuel supply is today commercial used. The common operational and constructional techniques in recent agricultural full scale biogas plants are modified from domestic sewage treatment plants where biogas production from organic waste is implemented since decades. However, apart from biowaste the organic substrates actually used in agricultural biogas plants are liquid manure from swine and cattlle as well as plant biomass in form of maize whole plant silage or grass silage exclusive of and also in combination.

If we talk about energy plants for biogas production foremost the maize whole plant is to mentioned. Moreover, the effective energy yields of maize silage are considerably higher than that of grass silage if both biogas substrates compared at the same level of fossil energy and nitrogen fertilizer input (Kelm et al, 2004). An overview of biogas substrates and energy yields are demonstrated in Table 2.

Table 2

Energy yields of different biogas substrates

substrate	**biogas yield** (m^3 t^{-1} FM[a)]	**methane yield** average (%)
maize silage	202	52
grass silage	172	54
rye GPS*	163	52
fodder beet	111	51
biowaste	100	61
poultry manure	80	60
sugar beet cuttings	67	72
swine manure	60	60
cattle manure	45	60
brewery grain	40	61
pig slurry	28	65
cattle slurry	25	60

GPS* = whole plant silage
FM = fresh mass
Table is modified from reference "**Fachagentur nachwachsende Rohstoffe, 2008**"

However, over the past years utilization of plant biomass for biogas production and energy recovery in form of electricity increases constantly in Austria. A similar trend could be also noticed in Germany since they started the Renewable Energy Sources Act (EEG), which guarantees a fixed payment for the produced electricity for 20

years (EEG, 2000). The electricity capacity of Austrian biogas plants range from 18 to 1000 kW_{el}. The energy and cost efficiency increases with the plant size and will be more or less profitable up to 250 kW_{el}. Nevertheless, an increase in plant size leads to a rise in cost regarding to substrate delivery to the plant and digestion residues from the plant. Furthermore, the availability of adequate substrate depends on the agricultural environment around the plant. However, an advantage in biogas production from renewable resources is the guaranteed payment for the so called green electricity for a plant size above 250 kW_{el} (Walla and Schneeberger, 2008).
An alternative way to the combustion of biogas in a CHP and conversion to heat and power, however, is the treatment of biogas to methane quality regarding natural gas and a further application as vehicle fuel.

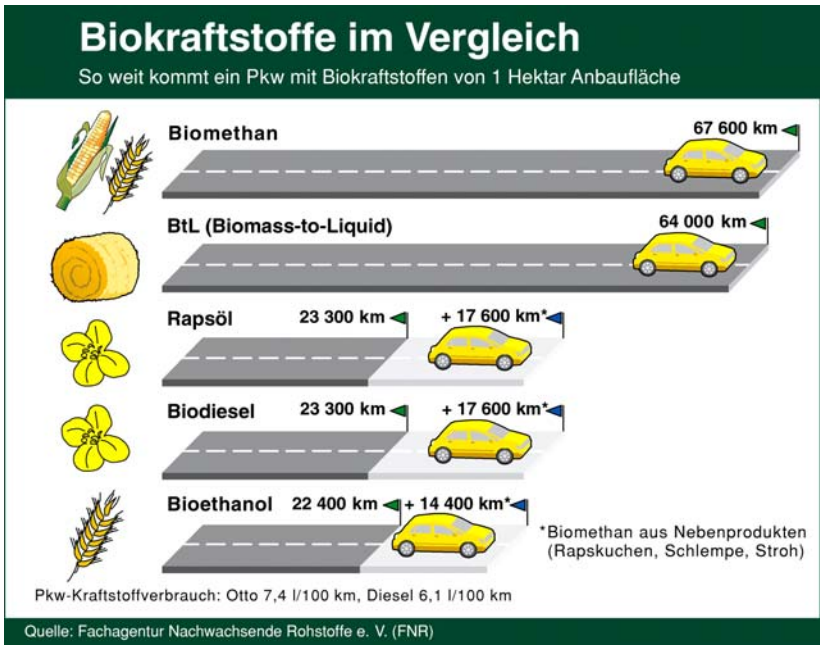

Figure 7. A comparison of varying bio-fuels. **Source:** "Fachagentur Nachwachsende Rohstoffe, Gülzower Fachgespräche, 29, 2008."

A comparison of different bio-fuels is illustrated in Fig. 7. This study demonstrates the energy yield of different bio-fuels which could be approximately gained from one hectare cultivable land. Moreover, calculated to an average fuel consumption of 7.4 L petrol and 6.1 L diesel a car could drive furthest with bio methane (Fig. 7). However, according to these results, anaerobic digestion of biomass and biogas production actually should be an alternative amongst other renewable resources for future energy supply.

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Objectives of the work

The aim of the present study was the mechanistic and process performance related investigation of the influence of heavy metals on anaerobic digestion and biogas production from plant biomass. Many full scale biogas plants are operating with maize silage as exclusive substrate facing problems after certain operation periods. For this reason the lab scale biogas fermentations were conducted also with maize silage and additionally with a defined model substrate for maize.

In the first part of the work consecutive anaerobic batch fermentations with maize silage and the defined model substrate, respectively and a selected trace metal solution were carried out. Based on the obtained results further batch experiments were performed with the defined model substrate only and donation of the selected trace metal solution. Furthermore batch trials were conducted with the single elements nickel, cobalt and molybdenum in comparison to the selected trace metal solution. In addition to these fermentation experiments bacterial and archaeal community analysis of batch trials with the defined model substrate and the selected trace metal solution and also trials with the defined model substrate and varying nickel concentrations were carried out. The community analysis was realized using SSCP (single strand conformation polymorphism analysis) and ARDRA (amplified rDNA restriction analysis).

The results of batch experiments suggested further investigations by means of semi-continuous anaerobic fermentations.

The semi-continuous bio reactors were actually conducted in two parallel operations. Experiments were executed with the defined model substrate, the selected trace metal solution, nickel and cobalt, respectively. The process stability was monitored by continuous measurements of the pH-value and the formation of volatile fatty acids which were analyzed by High Performance Liquid Chromatography (HPLC). The biogas quantity was measured using gas flow meters and the gas quality was detected with a special gas analyzer.

Publication I

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Influence of trace elements on methane formation from a synthetic model substrate for maize silage

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Abstract

The effect of a well-defined trace element solution and the elements nickel, cobalt and molybdenum on anaerobic digestion of a synthetic model substrate for maize silage was studied in batch reactor experiments at 35°C. The defined substrate (dS) consisted of xylan and starch as the main carbon source, urea as nitrogen source and phosphorus from a 0.1 M potassium phosphate buffer. Batch reactors were operated for 30 days with 1.5% organic dry matter (ODM) of inoculum sludge from a mono-maize biogas plant and 1% ODM of the defined substrate. Results showed an increase of methane yield of up to 30% upon addition of the trace element solution. With an addition of nickel at 10.6 μM, a final yield of 407 L kg^{-1} ODM was reached and an enhanced methane production by 25% at day 25 of operation was observed. Total elemination of nickel from the trace element solution highly decreased methane formation and process stability. Cobalt in a concentration range of 0.4 up to 2.0 μM increased the methane production by 10% approximately. Interestingly, addition of molybdenum did not significantly effect methane production.

Keywords: Trace elements, Xylan, Cobalt, Nickel, Molybdenum,

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1. Introduction

Production of biogas has been assessed from a variety of substrates ranging from organic household waste to more defined lignocellulosic substrates (Held et al, 2002; Staubmann et al, 1997). Methane-rich biogas produced through anaerobic digestion of maize silage is a carbon dioxide neutral and versatile renewable energy source. Bio-methane can be used in replacement for fossil fuels for vehicles and can also be utilised for power and heat production. For example, methane production through anaerobic digestion has been evaluated as one of the most energy-efficient and environmentally benign ways of producing vehicle biofuels (LBS, 2002).

The anaerobic digestion of biomass is a multi stage process involving different types of microorganisms. In addition to macronutrients such as carbon, nitrogen, phosphorus and sulphur, trace elements play a crucial role in the growth and metabolism of anaerobic microorganisms (Takashima and Speece, 1989; Goodwin et al., 1990) where they are essential for many physiological and biochemical processes. Many trace elements are metals (Zandvoort. et al, 2006). The effect was already investigated by using diverse synthetic media such as acetate in combination with nickel and cobalt (Kida et al., 2001). Specific trace metals such as cobalt, nickel, tungsten or molybdenum serve as cofactors in enzymes which are involved in the biochemistry of methane formation (Zandvoort. et al, 2006). Adequate availability of essential trace elements for the bacterial community is still a problem when single substrates rather than complex mixtures of materials are used for biogas production.

The bioavailability of trace elements for metabolic pathways of the anaerobic bacteria is in most cases not related to the total amount measured in the medium since only a fraction is present in solution (Oleszkiewicz and Sharma., 1990). Many parameters such as shifts in pH-value or temperature may lead to precipitatation and/or chelation

of trace elements thus reducing bioavailability (Zandvoort et al.; 2005; Mosey et al., 1971; Speece., 1996).

Especially biogas plants operating with maize silage as single substrate show consistently a lack of trace elements and consequently a decrease of biogas production (Clemens, 2007). This limitation leads to reduced methane yields and to considerable problems due to increasing process instability.

In the present work we studied the influence of various trace elements on anaerobic digestion and methane formation. A well-defined trace element solution and the single elements nickel, cobalt and molybdenum were added in different concentrations to anaerobic batch experiments. To exclude potential matrix effects from the maize silage, for the first time a synthetic model substrate for maize silage was used.

2. Methods

2.1. Inoculum

Biomass from 6 Austrian agricultural biogas plants was compared in order to select the digester sludge with lowest amount of trace elements as an inoculum for anaerobic batch experiments. Plant 6 was fed with 100% maize silage while the other biogas plants utilised maize silage as substrate in variations from 65 to 90% and additionally either liquid manure, grass silage, poultry manure and wheat residues or combinations of these substrates. Digester sludge from plant number 6 contained the lowest amounts of trace elements (Table 1) and was consequently utilised for all batch tests. The sludge was filtered to eliminate particles larger than 4 millimetres and diluted with distilled water to a concentration of 1.5% ODM.

2.2. Analytical methods

Macronutrients and trace metals were quantified in the inoculum sludge, in the maize silage and the defined substrate by ICP-OES (inductively coupled plasma - optical emission spectrometer, (Spectro Ciros Vision, Germany) (see also Table 1). Nitrogen was measured according to the method of Kjedahl with a Vapodest Vap 50, Gerhardt; Germany (data not shown). The pH-readings were conducted with a WTW pH 540 GLP pH-meter. The dry matter (DM) was determined at 105°C (Heraeus. Germany). The organic dry matter (ODM) was determined at 550°C (W. C. Heraeus HANAU. Germany).

Table 1

Macro- and micronutrient content in sludge from various Austrian biogas plants and in maize silage as quantified by ICP-OES

	macronutrients [mg kg^{-1} DM]						micronutrients [mg kg^{-1} DM]								
Element	P	K	Mg	Na	Ca	S	Cu	Cr	Ni	Zn	Fe	Co	Mn	Mo	Se
plant 1	19350	29700	5040	1240	7560	3700	24.4	4.2	3.6	110	1960	0.7	168	3	1.4
plant 2	19490	28530	3670	900	7640	3490	30	3.5	3.4	120	1170	<0.05	199	3.4	1
plant 3	28410	30180	6590	3070	17610	4390	147	7.2	5.5	220	1150	1.2	291	8.3	1.9
plant 4	19190	34010	5460	2110	13520	4370	34.9	4.9	3.9	220	1340	<0.05	170	16.5	1.7
plant 5	23460	29440	6030	3470	16670	4440	138.8	5.8	8.4	230	2630	1.6	264	6.1	1.7
plant 6	14170	28020	4120	680	9140	3610	23.9	1.9	3.1	150	1640	<0.05	159	1.1	1.4
MS	3590	8000	1250	50	1800	990	4.9	<0.05	0.7	22.4	80	<0.05	34.3	0.8	<0.05

Note: <0.05 = under detection limit of 0.05 mg kg-1 DM; MS = maize silage

2.3. Defined Substrate

A defined model substrate (dS) was developed mimicking the composition of maize. The main components of the maize plant are hemicellulose (i.e. xylan), starch and cellulose. Xylan from birchwood and starch from maize were thus used as basic carbon source in a ratio of 50% xylan 46% starch. The remaining 4% comes from urea. The urea was actually applied as nitrogen source while phosphorus was

supplied with a 0.1 M potassium phosphate buffer resulting in a C:N:P ratio of 125:5:1. All chemicals are purchased by Roth Chemicals. Germany.

2.4. Medium composition

The batch reactors were fed with a fermentation medium comprising 1% ODM from the defined substrate or from maize silage and 1.5% ODM from the inoculum. Trace metals were added as trace element solution (1 $mL \cdot L^{-1}$ fermentation medium). The trace element solution contained (µM) Fe^{2+} 7.5 ($FeCl_2 \cdot 4H_2O$), Zn^{2+} 0.5 ($ZnCl_2$), Mn^{2+} 0.5 ($MnCl_2 \cdot 4H_2O$), B^{3+} 0.1 (H_3BO_3), Co^{2+} 0.8 ($CoCl_2 \cdot 6H_2O$), Cu^{2+} 0.01 ($CuCl_2 \cdot 2H_2O$), Ni^{2+} 0.1 ($NiCl_2 \cdot 6H_20$), Se^{6+} 1.0 ($Na_2SeO_3 \cdot 5H_2O$), Mo^{6+} 0.15 ($Na_2MoO_4 \cdot 2H_2O$), W^{6+} 0.1 ($Na_2WO_4 \cdot 2H_2O$). Nickel, cobalt and molybdenum were also applied as single elements in concentrations as indicated below. Batch tests were started with a pH-value of 7.8 ± 0.2.

2.5. Experimental setup

All fermentations were carried out based on the guidelines of VDI for the “Fermentation of organic material” (Friedmann et al., 2004), modified according to DIN DEV 38414 S8 (1985). The trials were performed in 2000 ml reactors containing 1000 ml of fermentation medium. The reactors were mixed 8 times per day with a magnetic stirring system for 15 minutes. To remove carbon dioxide, generated biogas was washed with a 3 M sodium hydroxide solution. Ammonia and hydrogen sulphide were eliminated by an acidic solution containing 0.6 M $Na_2SO_4 \cdot 10\ H_2O$ and 0.5 M H_2SO_4. The displaced acidic solution was measured in a graduated cylinder and calculated to methane [L kg^{-1} ODM at STP]. The batch tests were conducted in triplicates at 35°C for a time period of 30 days. The results were compared with those

of control reactors operated with inoculum sludge exclusive of substrate and trace-element supplementation.

3. Results and discussion

3.1. Trace elements in biogas sludge and substrates

In a first step, trace elements present in the fermenter sludge from 6 different biogas plants and in maize silage were quantified. The lowest amount of nickel (3.1 mg kg-1 DM) was found in plant 6 (Fürstenfeld, Styria) which was fed with 100% maize silage (Table 1). Similarly, in this plant the lowest molybdenum content was detected with 1.1 mg kg-1 DM while the content of cobalt was below 0.05 mg kg-1 DM. Consequently, this sludge was considered to be the most suitable inoculum to study the influence of trace elements on anaerobic fermentation. 20 g DM L-1 from this sludge was used as inoculum for the batch trials thus resulting in a content of 1.05 µM nickel and 0.23 µM molybdenum. An additional introduction of 1.26 µM nickel and 0.32 µM molybdenum combined from inoculum and defined substrate in the fermentation trials was calculated based on analytical measurements. The measured concentration of cobalt was under the detection limit of 0.05 mg kg-1 DM in the defined substrate and maize silage respectively.

3.2. Implementation of a defined substrate and investigation of trace element effects on stabilisation and improvement of anaerobic fermentation

In a second step of the study on the influence of trace elements on methane production a model substrate for maize silage was developed. In a previous study Results from batch tests with maize silage and with the defined substrate indicates correlations of the methane formation amongst these two substrates (Fig. 1 a). The

addition of 1 ml trace element solution (1xTES) increased the methane yield by about 30% in both the tests with defined substrate and with maize silage. This is in agreement with results from Oleszkiewicz and Sharma (1990) who described the bioavailability of heavy metals like nickel, cobalt and molybdenum as an important factor for enhancement of methane production. However, upon addition of higher amounts of trace elements (5xTES) we observed just an increase of 10 to 13% of methane compared to the controls with defined substrate only. In consecutive experiment trace elements in the same amount as present in maize silage (see Table 1 “MS”) were added on top of the trace element solution (1xTES) to the defined substrate (Fig. 1 a: “1xTES-MSTE”). There was, however, no difference in methane production when compared to the batch test with 1xTES.

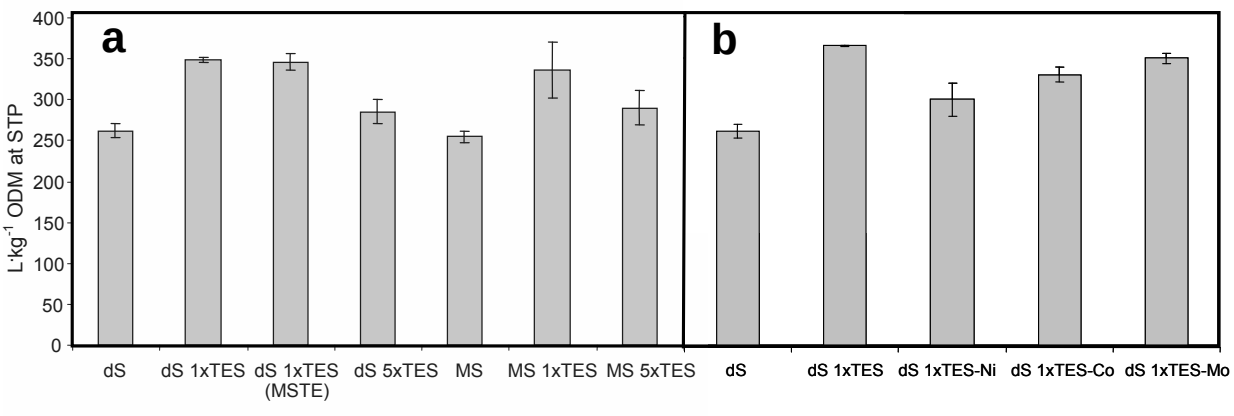

Fig. 1: Methane production from a defined model substrate (dS) and maize silage (MS) with different concentrations of trace elements (TES) **(a)**, and in the presence of trace elements (1xTES) and absence of Ni, Co and Mo, respectively **(b)**.

Batch experiments with the defined substrate and the trace element solution in the complete absence of either nickel, cobalt and molybdenum were also carried out. The results displayed in Fig. 1 b confirmed the large influence of trace elements on

methane production (increase by 30%) compared to tests with defined substrate only. The total elimination of nickel from the defined trace metal solution decreased the methane formation rapidly and leads to significantly lower methane yields by about 18% compared to batch trials with defined substrate supplemented with all trace elements (1xTES). Thus, nickel resulting from the inoculum sludge (1.05µM) is not sufficient or only bio-available to a limited extend. Furthermore, instability in the fermentation process was detected at low nickel concentrations. The major role of nickel in anaerobic digestion was reported in many studies, for instance Zandvoort et al. (2006) described nickel as cofactor in the key enzyme Methyl-CoM-reductase. Furthermore Mulrooney and Hausinger (2003) described the uptake and utilisation of nickel by microorganisms which are also involved in anerobic digestion as a highly complex multi stage process. However, a lack of cobalt in the added trace metal solution resulted also in a decrease of process stability and a drop of methane production by approximately 10% compared to the trials with 1xTES. In a previous study with synthetic wastewater and acetate as carbon source, Kida et al. (2001) observed a 13 fold increase of methane production after addition of 10 µM nickel and 4 µM cobalt.

The absence of molybdenum in the trace metal solution did, however, not have a significant effect on methane production. Molybdenum was described by Zandvoort et al. (2006) as a cofactor in enzymes which are involved in the formate conversion during methane generation. Furthermore, in a study from May et al. (1988) the substitution of molybdenum by tungsten in some methanogenic bacteria was demonstrated.

3.3. Evaluation of the concentration optimum of nickel, cobalt and molybdenum

In a number of serial batch experiments a broad range of concentrations for the elements nickel, cobalt and molybdenum was tested. In Table 2, methane yields in the presence of varying concentrations of nickel, cobalt and molybdenum from a low level up to inhibition of methane production are listed. Batch trials with nickel showed the best methane performance between 0.4 and 10.6 µM. Furthermore the addition of nickel in a concentration of 10.6 µM led to a continuous increase of methane yields and final yields of methane were reached 5 days faster compared to trials with defined substrate only (data not shown). It seems that an increase above this concentration had already an inhibitory influence on anaerobic digestion and leads to a fast drop down of methane production. Similarly, Williams et al. (1986) described an increase of biogas production after addition of 10 µM L^{-1} nickel in a laboratory poultry waste digester. An increase of the nickel concentration from 10.6 µM to 17.4 µM decreased the methane yield by 40%. A further 10 fold increase of the concentration of nickel to 171 µM reduced the methane production only 25% compared to batch trials with 17.4 µM nickel.

Table 2

Methane production from a defined model substrate for maize silage in presence of different concentration of nickel (a), cobalt (b) and molybdenum (c)

nickel		cobalt		molybdenum	
[µM]	methane yield ($L.kg^{-1}$ ODM at STP)	[µM]	methane yield ($L.kg^{-1}$ ODM at STP)	[µM]	methane yield ($L.kg^{-1}$ ODM at STP)
dS	342	dS	335	dS	370
0.4	399	0.4	341	1.7	375
1.4	386	0.5	344	2.3	378
10.6	407	2.0	371	6.4	374
17.4	245	17	320	11.6	383
171	188	170	288	53	390
				521	371
				1042	345

Note: “dS” demonstrate batch trials with defined substrate as control

The influence of cobalt on methane formation was determined in a concentration range between 0.4 and 170 µM. Batch tests with addition of cobalt showed best methane production with 2 µM, but already small fluctuations of methane formation between 0.2 and 17µM. For instance, a stimulatory effect of cobalt on anaerobic fermentation of grass-clover silage was clearly shown at concentrations about 0.4 µM (Jarvis et al., 1997). Cobalt stimulates methane generation in smaller amounts in contrast to nickel. Consequently, inhibitory effects of nickel and cobalt at concentrations of 17.4, and 170 µM respectively, were determined. Inhibition of methane formation by heavy metals was also described, for instance Zayed and Winter (2000) reported decreasing methane performance by addition of 75 µM nickel for anaerobic digestion of whey.

The investigations of molybdenum on anaerobic digestion of our defined substrate demonstrated the best methane production between 1.7 and 521 µM with a peak of methane activity at 53 µM (Table 2). Murray and Berg (1981) also reported that an addition of molybdenum (50 nM) was only slightly stimulatory when added in combination with both nickel and cobalt. However, the highest added amount of molybdenum of 1042 µM indicated no clear inhibitory effect on the anaerobic fermentation of the defined substrate.

5. Conclusion

In batch digestion of maize silage and a defined model substrate an increase of methane yields by up to 30% upon addition of a mixture of trace elements (TES) as well as with nickel alone was demonstrated. Cobalt enhanced methane production by up to 15%. Molybdenum alone did not significantly enhance methane formation. For a more detailed investigation of the effect of molybdenum on anaerobic digestion

further batch tests exclusive of the elements cobalt, nickel and also tungsten should be carried out. Batch tests with a defined model substrate were demonstrated to be a suitable system for a rapid estimation of the effect of trace elements on biogas production. Long-term effects nickel, cobalt and molybdenum on the biogas process should, however, be studied in continuous or semicontinuous reactors.

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Manuscript I

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Characterization of an anaerobic population digesting a model substrate for maize in the presence of trace metals

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Abstract

The influence of a defined trace metal solution and additionally Ni^{2+} on anaerobic digestion of biomass was investigated. A novel synthetic model substrate was designed consisting of cellulose, starch and urea as carbon and nitrogen source in a ratio mimicking the basic composition of maize silage. Two independent batch fermentations were carried out over 21 days with the synthetic model substrate in the presence of the trace metal solution. Particularly an increase in nickel concentrations (17 and 34 µM) enhanced methane formation by up to 20%. This increased activity was also corroborated by fluorescence microscopy measurements based on cofactor F_{420}. The eubacterial and methanogenic population was characterized with SSCP (single strand conformational polymorphism) and ARDRA (amplified 16S rDNA restriction analysis) of 16S rRNA genes amplified by different primer systems. Nearly the half of the analyzed bacteria were identified as *Firmicutes* while 70% in this phylum belonged to the class of *Clostridiales* and 30% to the class of *Bacilli*. *Bacteroides* and uncultured bacteria represented each a quarter of the analyzed community. Methanogenic archaea were investigated with ARDRA, too. The

hydrogenotrophic *Methanoculleus sp*. was the dominant genus which is commonly described for maize digestion thus confirming the value of the model substrate.

Keywords: Anaerobic digestion, Biogas, Cellulose, Nickel, SSCP

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1. Introduction

The environmental impact of anaerobic digestion and methane formation is related to its ecological role, on the one hand accumulation and effect as a greenhouse gas, on the other hand an application for conversion of biomass agricultural residues and biowaste into energy (Chynoweth, 1996). Production of biogas from a variety of substrates ranging from organic household waste to more defined lignocellulosic substrates is a carbon dioxide neutral and versatile renewable energy source (Held et al., 2002; Staubmann et al., 1997). Especially maize silage from the whole maize plant as energy crop has a high potential for biogas production. Anaerobic degradation of organic matter and methane formation is a multi step process carried out by a consortium of microorganisms. The production of methane requires a trophic chain of at least three interacting metabolic groups of strictly anaerobic microbes (Ferry, 1997). First of all the hetero-fermentative group reduce cellulose and other complex molecules into volatile fatty acids (e.g. acetate, propionate), CO_2, and H_2. The syntrophic bacteria further metabolize the alcohols and fatty acids into acetate,

H_2, and CO_2, in which acetate, H_2, and CO_2 finally serve as substrates for the methanogenic archaea (Guedon et al., 2002). A detailed knowledge about the composition of these microbial communities participating in the degradation process of biomass will be essential to understanding and improve the biogas-forming process (Klocke et al., 2007). In addition to microbial community studies it is known from previous investigations that various trace metals play an important role in growth and biochemical processes of the methane producing microorganisms. (Takashima and Speece, 1989; Goodwin et al., 1990). Specific trace metals such as cobalt, nickel, tungsten or molybdenum serve as cofactors in enzymes which are involved in the biochemistry of methane formation (Zandvoort. et al, 2006). For example, all methanogenic pathways converge to the enzymatic reduction of methyl coenzyme M to methane. This reduction is catalyzed by the Methyl-coenzyme M reductase complex, which includes a nickel containing cofactor called F_{430} (Friedman et al., 1990). Nevertheless, availability of essential trace elements in optimal amounts is still a problem when single substrates rather than complex mixtures of organic matter are used for biogas production. The bioavailability of trace elements for metabolic pathways of the archaeal and bacterial community is in most cases not related to the total amount measured in the medium since only a fraction is present in solution (Oleszkiewicz and Sharma, 1990). Shifts in pH-value or temperature may lead to metal precipitatation and/or chelation which induce to a reduced bioavailability of these trace metals. A limitation of essential trace metals can lead to reduced methane yields and to considerable problems due to increasing process instability (Zandvoort et al.; 2005; Mosey et al., 1971; Speece., 1996).

The objective of the present study was to characterize an anaerobic microbial community digesting a cellulose/starch based model substrate in the presence of trace elements. Both the replacement of real substrates (i.e. maize) with synthetic

components as well as addition of trace elements could have an impact of microbial populations which has not been studied yet in detail. A combination of SSCP and ARDRA was used to illustrate the population developing under these conditions.

2. Materials and methods

2.1. Liquid and solid media

The used LB media consisted of Peptone 16 g L^{-1}, Yeast extract 10 g L^{-1}, NaCl 5 g L^{-1} and 2xTY media Peptone 10 g L^{-1}, Yeast extract 5 g L^{-1}, NaCl 5 g L^{-1}, respectively. For the LBA agar, LB media was added with 14 g L^{-1} Agar Agar and 100 µg mL^{-1} ampicillin. The LBA/X-Gal-agar was assembled like LBA agar with additionally 40 µg mL^{-1} X-Gal (bromo-chloro-indolyl-galactopyranoside). All media were prepared with deionised water and autoclaved. Ampicillin solution was sterile filtrated. All chemicals were purchased by Roth and Merck (Germany).

2.2. Batch tests

2.2.1. Model substrate and fermentation medium

A new synthetic model substrate (dS) was developed following the composition of maize. Microcrystalline cellulose and starch from maize were used as carbon source in a mix of 50% cellulose and 46% starch, besides urea as a nitrogen source accounting for the remaining 4%. In addition phosphorus was supplied with a 0.1 M potassium phosphate buffer resulting in a C:N:P ratio of 125:5:1. All chemicals are purchased by Roth Chemicals, Germany. The batch reactors were fed with a fermentation medium comprising 1% ODM from the defined model substrate and 1.5% ODM from an inoculum sludge (biogas plant Fürstenfeld, Styria). Trace metals

were added as trace element solution (1 mL L^{-1} fermentation medium). The trace element solution contained (µM) Fe^{2+} 7.5 ($FeCl_2 \cdot 4H_2O$), Zn^{2+} 0.5 ($ZnCl_2$), Mn^{2+} 0.5 ($MnCl_2 \cdot 4H_2O$), B^{3+} 0.1 (H_3BO_3), Co^{2+} 0.8 ($CoCl_2 \cdot 6H_2O$), Cu^{2+} 0.01 ($CuCl_2 \cdot 2H_2O$), Ni^{2+} 0.1 ($NiCl_2 \cdot 6H_20$), Se^{6+} 1.0 ($Na_2SeO_3 \cdot 5H_2O$), Mo^{6+} 0.15 ($Na_2MoO_4 \cdot 2H_2O$), W^{6+} 0.1 ($Na_2WO_4 \cdot 2H_2O$). Additional nickel was also applied as $NiCl_2 \cdot 6H_20$ in concentrations as indicated below. Batch tests were started with a pH-value of 7.8 ± 0.2.

2.2.2. Experimental set up

Batch tests were conducted according to Friedmann et al., 2004 and modified as described in DIN DEV 38414 S8 (1985). The batch fermentations were performed in 2 L glass reactors with a volume of 1 L of the fermentation medium. Carbon dioxide was removed from the biogas with a 3 M sodium hydroxide solution. Ammonia and hydrogen sulphide were eliminated by an acidic solution containing 0.6 M $Na_2SO_4 \cdot 10$ H_2O and 0.5 M H_2SO_4. The displaced acidic solution was measured in a graduated cylinder and calculated to the produced methane. The reactors were mixed with a magnetic stirring system for 15 minutes every 3 hours. The batch trials were carried out in triplicates at 35°C for a time period of 21 days. Results from control reactor were compared with reactors inclusive of defined model substrate and appropriate trace metals.

2.3. Analytical measurements

ICP-OES, Spectro Ciros Vision, Germany was used for the quantification of macronutrients and trace metals in the inoculum sludge and the defined model substrate. Nitrogen was measured according to the method of Kjedahl with a Vapodest Vap 50, Gerhardt; Germany (data not shown). The pH-readings were conducted with a WTW pH 540 GLP pH-meter. The dry matter (DM) was determined

at 105°C (Heraeus. Germany). The organic dry matter (ODM) was determined at 550°C (W. C. Heraeus HANAU. Germany). Volatile fatty acids (VFAs) were measured by HPLC. Thus 1 g of sludge from batch fermentation was centrifuged for 10 min at 16,000 g and the supernatant was collected. An aliquot of the supernatant was pre-treated according to Carrez precipitation to remove proteins and fat components. For HPLC performance a Hewlett Packard HPLC System 1100 was used. The system was equipped with aTRANSGENOMIC, ICSep ION-300, Art Nr. ICE-99-9850 column and WAGNER LÖFFLER, ICSep ION-300, Art Nr. CH0-0800 column respectively. As pre-column TRANSGENOMIC, GC801/C, Art Nr. ICE-99-2364 and WAGNER LÖFFLER, Interaction Replacement Cart. GC-801/C,24*4,0mm, Art Nr. CH0-0831 respectively were used. Measurements were carried out at following operating conditions: 0.005 M H_2SO_4 as mobile phase and a flow rate of 1 mL min^{-1}, injection volume 40 µL, column temperature 42°C. For reporting of chromatograms special software was used (HP chemstation).

2.4. DNA extraction and PCR amplification

Samples of biogas sludge from batch reactors were obtained on days 1, 7, 14, 21 and 28 for gene-based analyses. Therefore in each case 1 g of the biogas sludge were centrifuged for 15 min at 16,000 g and 4°C with a Hermle Z300K (Hermle Labortechnik, Germany). The supernatant was discarded and the pellet dissolved in 1mL extraction buffer consisting of 100 mM Tris, 100 mM EDTA, 100 mM NaCl, 1% Polyvinylpyrrolidon and 2% SDS. Total genomic DNA was actually extracted according to the protocol of Martin-Laurent et al. (2001). Bacterial 16S rRNA gene target for SSCP analysis was amplified with forward primer Com 1 (5'-CAGCAGCCGCGGTAATAC-3'; (Schwieger and Tebbe, 1998) and reverse primer 927r (5'-CCCGTCAATTYMTTTGAGTT-3'; Lieber et al., 2002). Primer 927r was

phosphorylated for single strand digestion. The 50 µL reaction mixture consists of 10 µL Taq&Go (MP Biomedicals Europe), 1.67 µL 50 mM $MgCl_2$, 2 µL of forward and reverse primer respectively (5 pmol μL^{-1}), 1 µL template DNA, 33.3 µL H_20 (Roth, Germany). PCR was performed with an initial denaturation at 94°C for 3 min, 35 amplification cycles (60 sec at 94°C, 60 sec at 50°C, and 70 sec at 72°C), and a final elongation at 72°C for 5 min.

Archaeal and bacterial 16S rRNA genes for ARDRA analysis were amplified with forward primer 21F (5'-TTCCGGTTGATCCYGCCGGA-3'; De Long 1992) and reverse primer 958R (5'-YCCGGCGTTGAMTCCAATT-3'; De Long 1992), and forward primer 27F (5'-GAGTTTGATCCTGGCTCAG-3'; Liesack et al., 1991) and reverse primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3'; Lane et al., 1991,), respectively. In each case, a 20 µL reaction mixture for archael and bacterial DNA PCR-amplification was conducted contained 4 µL Taq&Go (MP Biomedicals Europe), 1. µL 50 mM $MgCl_2$, 1 µL of archael and bacterial forward and reverse primer respectively (5 pmol μL^{-1}), 1 µL template DNA, 13 µL H_20 (Roth, Germany). The PCR program for archael DNA amplification consisted of a initial denaturation at 95°C for 5 min, 29 cycles (30 sec at 95°C, 30 sec at 55°C and 90 sec at 72°C), and a final elongation at 72°C for 10 min. Further PCR for bacterial DNA amplification consisted of initial denaturation at 95°C for 5 min, 9 cycles (30 sec at 95°C, 30 sec at 52°C and 100 sec at 72°C), 19 cycles (30 sec at 95°C, 30 sec at 52°C and annealing at 72°C for 30 sec with an increase of 10 sec every cycle) and a final elongation at 72°C for 5 min.

The Colony-PCR of positive clones was performed with the universal sequencing primers rsp (5'-CAGGAAACAGCTATGACC-3') and usp (5'-GTAAAACGACGGCCAGT-3'). PCR reaction mix was done in a volume of 20 µL as described for archael and bacterial DNA amplification. The PCR program consisted

of a initial denaturation at 95°C for 5 min, 29 cycles (30 sec at 95°C, 30 sec at 54°C and 60 sec at 72°C), and a final elongation at 72°C for 10 min.

The products from all PCRs were purified with the Wizard® SV Gel and PCR Clean-Up System kit (Promega). Furthermore all PCR products were examined by electrophoresis on 0.8% agarose gels, viewing on a UV transillumination table to determine correct amplicon size.

2.5. SSCP and ARDRA analysis

SSCP analysis of amplified bacterial 16S rRNA gene fragments was realised according to Schwieger and Tebbe (1998). Exonuclease digestion of purified PCR products was performed with a λ-Exonuclease, 12 U (New England Biolabs, Germany) at 37°C for 1 h, followed by an addition of 50% (v/v) loading buffer (95% deionised formamide, 10 mM NaOH, 0.025% (w/v) bromophenol blue), a denaturation step at 98°C for 3 min and a regeneration step on ice for 5 min. Electrophoretic separation of DNA single strands was performed on a 8% polyacrylamide gel and a 1 x TBE buffer (89 mM TRIS, 89 mM boric acid, 2 mM Na_2EDTA) for 26 h at 26°C, 400V and 50mA, using a TGGE MAXI system (Biometra). After silver-staining according to Bassam et al. (1991), gels were digitalised using a transillumination scanner and bands of interest were excised with a sterile scalpel for following sequence analysis. DNA was eluted from gel slices through incubation in sterile elution buffer (5 M ammonium acetate, 10 mM magnesium acetate, 1 mM Na_2EDTA, 0.1% SDS, pH 8.0) at 37°C for 5 h following the “crush and soak” extraction method previously described by Sambrook et al. (1989). The eluted DNA was amplified again using the same primer pairs for the SSCP analysis as described above. The sequencing was done at Eurofins MWG Operon, Germany. A minimum DNA concentration of 5ng μL^{-1} was used.

ARDRA analysis of amplified bacterial and archael 16S rRNA gene fragments was performed according to Vaneechoutte et al. (1992). The obtained PCR amplicons from PCR with archael and bacterial primers respectively (2.4.) were ligated into the pGEM®-T vector system I (Promega) and used to transform elcetrocompetent *E. coli* DH5α cells with a Bio-Rad MicroPulser™ by following the manufacturer's instructions. Clone libraries were generated, by growing regenerated cells undiluted and 10^{-1} diluted on LBA/X-Gal plates (2.1.) and incubated over night at 37°C. A Colony-PCR as described above was done from positive, white clones. Amplification products were controlled on a 0.8% agarose gel to determine the concentration of the DNA for following digestion with restriction endonuclease *Hha*I (Fermentas) at 37°C for 3 h. Resultant DNA fragments were separated electrophoretically for 3 h and 120 V on 1.5% 0.5 TBE agarose gels and afterwards stained in a ethidium bromide solution for 20 min. Operational taxonomic units (OTUs) were identified manually and additionally the classification was done with GelCompare© (Applied Maths, version 4.2) based on restriction cleavage patterns and clones representing the OTUs selected for sequencing. The sequencing was also done at Eurofins MWG Operon, Germany.

2.6. Fluorescence microscopy

Fluorescent microscopic analyses were carried out to determine the activity of methanogenic bacteria. Samples were taken on day 1, 7, 14 and 21 and investigated immediately under a Leitz Laborlux S fluorescence microscope. A 100 W high-pressure mercury lamp was used with a 460nm longpass filter for blue excitation. 10 µL of each sample were fixed on a microscope slide. The samples were observed with a 1000-fold magnification. For documentation a Nikon D70 camera and the software Camera control Pro were used.

3. Results and discussion

3.1. Performance of anaerobic batch tests

Two independent batch fermentations with a novel model substrate for maize silage and addition of trace metals and supplementary nickel were conducted. A basic nickel concentration of 2 µM was resulted in the batch fermentations from the utilized inoculum sludge. Additionally nickel concentrations from 5 - 17 µM (batch 1) and 8.5 - 34 µM (batch 2) were analyzed (Table 1). Addition of the trace metal solution increased methane production by 4% in batch 1 and 6% in batch 2 respectively. In a previous study Murray and Berg (1981) reported also an increase of methane production of more than 10% after addition of a trace metal composition including nickel, cobalt and molybdenum. Addition of 5 µM nickel to fermentations in batch 1 induced a methane enhancement of 8% compared to trials exclusive of defined substrate and which increased until a plateau at 17 µM nickel. Therefore, a second series of batch fermentations was conducted to evaluate higher nickel concentrations. However, additions of up to 34 µM nickel did not further increase methane production (Table 1). The obtained results from the conducted batch fermentations demonstrated the best performance and methane production at a nickel dosage of 17 µM in batch 1 with a methane increase of 14% and batch 2 up to 20%. These increases are in a similar range as previously reported for a xylan based substrate (Pobeheim et al., 2010). However, in this previous study the optimum effect was reached at a slightly lower nickel concentration of 10.6 µM. Similarly, in a study of Williams et al. (1986) an addition of 10 µM nickel to a chicken manure digester significantly stimulated biogas production, while nickel was present in the effluent (253 µM) before supplementary “fresh” nickel was added.

Moreover we determined formation and degradation of volatile fatty acids (VFA`s). Samples from day 1, 7, 14 and 21 were taken and measurements from acetic and propionic acid were analyzed (Table 2). At day 7 an increase of acetic acid between 3187 mg kg^{-1} FM (reactor 7) and 2240 mg kg^{-1} FM (reactor 8) and propionic acid between 1635 mg kg^{-1} FM (reactor 5) and 1377 mg kg^{-1} FM (reactor 8) were consistent with the drop of pH-value from 7.8 ± 0.2 (day 1) to 6.8 ± 0.1. Batch trials with addition of 17µM nickel (reactor 6) showed a constant degradation of acetic acid to 640 mg kg^{-1} FM and lowest amount of propionic acid (146 mg kg^{-1} FM) on day 21. Furthermore a pH-value of 7.6 in relation to remaining trials with pH-values between 7.35 and 7.5 were measured. Accumulation of propionic acid is an indication for process instability and could result from limited activity of synthrophic and / or methane bacteria. Alternatively inhibition of propionate degradation by propionate and acetate could happen due to the undissociated acid forms of these compounds as observed by Fukuzaki et al. (1990).

Table 1

Effect of nickel on methane yield in anaerobic digestion of a maize model substrate

		batch 1		batch 2	
reactor	substrates	NL[c] CH_4	methane yield (%)	NL CH_4	methane yield (%)
1	dS[a]	225 ±10	100	341 ±2	100
2	dS+TES[b]	235 ±12	104	375 ±14	110
3	dS+TES + 5.0 µM	243 ±10	108		
4	dS+TES + 8.5 µM	221 ±33	98	326 ±38	96
5	dS+TES + 13 µM	253 ±17	113	377 ±14	111
6	dS+TES + 17 µM	255 ±0	114	388 ±17	114
7	dS+TES + 26 µM			337 ±40	99
8	dS+TES + 34 µM			386 ±16	113

note: 5.0 – 34 µM additionally nickel to anaerobic batch trials
[a] defined model substrate
[b] trace element solution
[c] [L kg^{-1} ODM at STP].

Table 2

Acetic and propionic acid production during anaerobic digestion of a maize model substrate and different nickel concentrations over 21 days

	acetic acid [mg kg $^{-1}$ FM[a]]						
	reactor						
sample (days)	1	2	4	5	6	7	8
1	124	131	121	127	126	134	134
7	2241	2377	2648	2641	2576	3187	2440
14	3236	3488	3337	3156	3065	172	2108
21	908	819	579	347	640	35	467
	propionic acid [mg kg $^{-1}$ FM]						
1	0	0	0	0	0	0	0
7	1545	1509	1503	1635	1546	1532	1377
14	1461	1478	1493	1649	1313	1650	1387
21	1152	795	449	1547	146	1642	769

note: The reactors has the same number and contents as described in Table 1.
[a] fresh mass of fermentation medium

3.2. Analysis of eubacterial community by SSCP

SSCP analysis of 16S rRNA genes was used to characterize the anaerobic bacterial community developed on the maize model substrates in presence and absence of trace elements. SSCP analysis is a semi-quantitative method while each band on the gel should represent one species. For this a 400bp long fragment of the variable region V4-V5 **(**Schmalenberger et al., 2001) of the bacterial 16S rDNA was amplified. A couple of diverse bands were excised from SSCP gels, re-amplified and sequenced.

Table 3

Summary of sequences from excised SSCP gel bands identified by searching the NCBI BLAST database.

band	sampling point	species	accession number
		batch 1	
1	R 5 day 21	Unc. Bacterium	AB248650.1
2	R 5 day 21	Unc. Bacterium	FJ205858.1
3	R 5 day 21	*Clostridium sp.*	DQ168250.1
4	R 1 day 1	*Unc.Arcobacter sp.*	EU403949.1
5	R 5 day 7	*Clostridium sp.*	AY330125.1
6	R 6 day 14	*Sedimentibacter sp.*	EF464626.1
		batch 2	
7	R 6 day 7	*Clostridium sp.*	AY330125.1
8	R 7 day 7	*Clostridium sp.*	DQ168269.1
9	R 8 day 14	*Clostridium sp.*	DQ168299.1
10	R 4 day 14	*Clostridium sp.*	AB286232.1
11	R 7 day 14	Unc. Bacterium	AB248650.1
12	R 7 day 14	*Proteiniphilum acetatigenes*	AY742226.1
13	R 5 day 14	Unc. Clostridiales	EU741667.1
14	R 6 day 14	Unc. Bacterium	FJ205864.1
15	R 8 day 1	*Clostridium sp.*	AB288660.1

From the first batch fermentation with nickel concentrations from 5 to 17 µM 6 bands could be sequenced and assigned (Fig. 1 a). As reported in Table 3 band 1 and 2 were assigned as uncultured bacterium, band 3 and 5 as *Clostridium sp*., band 4 as *Arcobacter sp*. and band 6 as *Sedimentibacter sp*. *Clostridia* are gram-positive, rod-shaped, anaerobic and endospore forming bacteria. Saccharolytic *Clostridia* are known to degrade polysaccharides like cellulose and starch (where the substrate in this study is based on) leading to monosaccharides (Zhang et al., 1994). One strain belonging to *Clostridia* (B3) started as a weak band and became dominant after day 7 (Fig 1a). Another representative band (B5) showed the strongest appearance at day 7, too. Obviously these hydrolytic bacteria degraded the polysaccharide substrates yielding intermediates for methane production. This is also indicated by a drop down of the pH-value and increased VFA concentrations at day 7 (Table 2). *Arcobacter spp*. belongs to the family of *Campylobacteriaceae*. They are gram-

negative, rod-shaped bacteria and are able to grow under aerobic and anaerobic conditions. These bacteria are common in poultry meat and manure (HO Hoa et al., 2006). This bacterium was detected at the beginning of the fermentation (band 4), while the corresponding band became weaker at day 7 and disappeared after day 14. This nicely demonstrates the power of this analysis method since indeed poultry manure was used as inoculum sludge for the batch fermentations. However, the disappearance of these bacteria indicates that they play no dominant role in the degradation of polysaccharides and were displaced after one week by other hydrolytic bacteria.

Sedimentibacter sp. is counted to the order of Clostridiales. They are gram-positive, anaerobic, endospore forming bacteria. These bacteria utilize amino acids and pyruvate as substrates and metabolise acetate and butyrate. They do not produce hydrogen and carbohydrates are not fermented. The genus has a high similarity to Clostridia (Breitenstein et al., 2002). The corresponding band in Fig 1a was dominant at day 14 when butyric and isobutyric acids were present at their highest amount with 80 mg kg^{-1} FM and 100 mg kg^{-1} FM for butyric and isobutyric acid respectively.

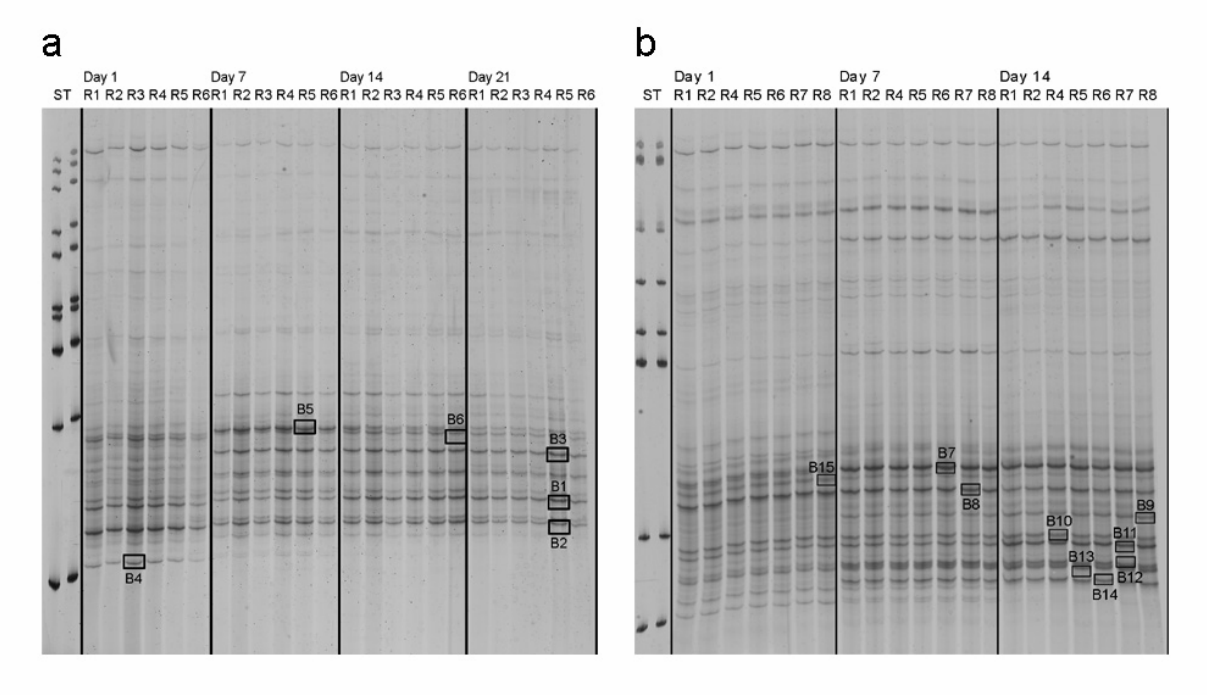

Fig. 1. SSCP gels of fragments of the 16S rDNA extracted from a batch anaerobic fermentation of a maize model substrate **(a)** with additionally nickel concentrations from 5 – 17 µM and second fermentation **(b)** from 8.5 – 34 µM. The framed bands were cut out and DNA was amplified for sequencing (Table 3). Band patterns show a similar behaviour on both gels. 1 kb standard (ST) was used.

Moreover 9 bands were considered for sequencing from the second batch fermentation (8.5 - 34 µM nickel). Thereof 5 bands corresponded to *Clostridium sp*., 2 bands to uncultured bacteria, one to uncultured Clostridiales and *Proteiniphilum acetatigenes* respectively and one band was identified as an uncultured Bacteroidetes (Table 3). *Proteiniphilum* are gram-positive, anaerobic, non-spore forming bacteria. They utilize yeast extract, peptone, pyruvate, glycine and L-arginine as carbon and energy sources. Previously, representatives were isolated from a triculture degrading propionate to acetate and methane. They are symbiotic bacteria of the acetogenesis which is an important process to degrade the produced volatile fatty acids to acetate for the following methanogenesis (Chen and Dong, 2005). The

corresponding SSCP gel band (B 12) showed a weak appearance on day 1 and became dominant at day 7 (Fig. 1b). Members of *Clostridium sp*. are important bacteria in hydrolysis. Corresponding bands showed different appearances over time (Fig 1a and b). For example band 8 was dominant at the beginning and got weaker till day 21. The detected *Clostridium sp*. in band 10 seems constant over time. This could mean that individual representatives have a different substrate specificity ranging from polymers to smaller molecules.

In summary, manual analysis of the SSCP gels obtained over the duration of the batch fermentations was carried out. The presence of certain organisms deducted from the evolvement or diminishment of different bands nicely reflected their role in the microbial process. However, a clear influence of different nickel concentrations was not seen.

Additionally, a clustering of the band patterns from the first batch fermentation was done with GelCompare© (Applied Maths, version 4.2). The clustering method was UPGMA (Unweighted Pair Group Method with Arithmetic mean) and Clustering Correlation. With this method the program compares the position and the intensity of the bands.

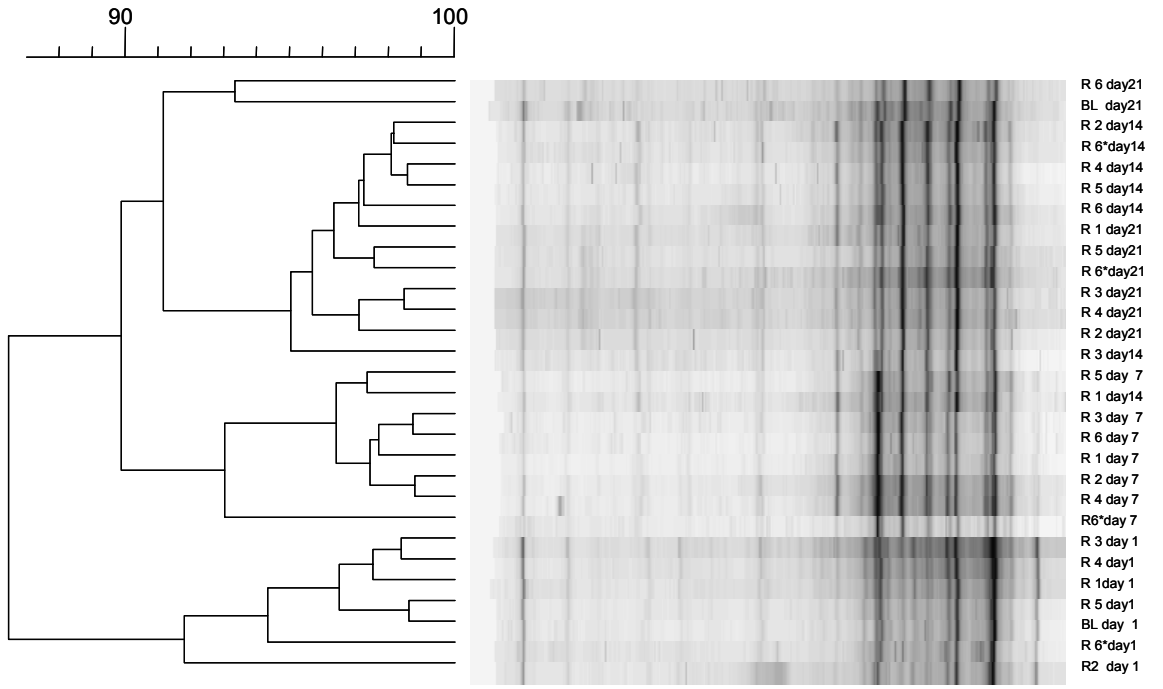

Fig. 2. Clustering of band patterns from the first batch anaerobic fermentation of a maize model substrate in the presence of nickel concentrations from 5 - 17 µM. The similarity of different clusters are indicated on the bar. BL means fermentations with inoculum sludge only, R* are parallel reactor (R 6) with 17 µM nickel.

The program generated three major clusters which show at least 87% similarity (Fig. 2). The first cluster consists of batch trails from day 1. These represent the community of the seeding sludge hence no significant difference can be seen in the band patterns. The second cluster represents all batch trials of day 7 inclusive of R 1 day 14. This means that there has been a change in the community within the first seven days. The third cluster represents the batch fermentations of day 14 and day 21. They have a similarity of 95%. This indicates that no major change in the bacterial community appeared after day 14. The reason for this could be that the substrate is already consumed and the major community of hydrolyzing Clostridia is stable. R 6 day 21 and blank day 21 were not classified in a distinct cluster. Further

no significant changes in bacterial abundance between different nickel concentrations could be investigated.

3.3 Eubacterial and methanogenic population dynamics investigation by ARDRA analysis

In addition to the SSCP analysis for the first anaerobic batch fermentation an ARDRA analysis from the trial with addition of 17 µM nickel was done from samples of day 1 and day 21. The community DNA was amplified with bacterial and archaeal primers, respectively. For each of the two sampling points 100 clones were used. For day 1 just 68 and for day 21 90 clones showed a positive result in the following PCR amplification.

Table 4

Characterisation of an anaerobic microbial population during degradation of a maize model substrate: Occurrence and specification of the different OTUs according to ARDRA analysis

	day 1	day 21						
OTU	%	%	phylum	class	order	family	genus	species
1	19.1	11.1						Unc. Bacterium
2	2.9	1.1	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	*Butyrivibrio*	*Butyrivibrio*
3	5.9	15.6	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Leuconostoc*	*Leuconostoc*
4	2.9	4.4	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	
5	4.4	1.1	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*Clostridium sp.*
6	0.0	2.2	Firmicutes	Clostridia	Clostridiales	Incertae sedis XI	*Sedimentibacter*	*Sedimentibacter*
7	1.5	2.2	Firmicutes					
8	8.8	4.4	Bacteroidetes	Bacteroidetes	Bacteroidales	Bacteroidaceae	*Tannerella*	*Tannerella*
9	1.5	5.6						Unc. Bacterium
10	0.0	1.1	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*Bacillus sp.*
11	5.9	12.2	Unc. Bacteroidetes					
12	4.4	2.2						Unc. Bacterium
13	4.4	4.4	Bacteroidetes	Bacteroidetes	Bacteroidales	Bacteroidaceae	*Bacteroides*	*Bacteroides acidifaciens*
14	0.0	2.2	Unc. Bacteroidetes					
15	7.4	2.2	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*Clostridium*
16	4.4	2.2	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	*Pelospora*	
						Peptococcaceae	*Desulfotomaculum*	
							Pelotomaculum	
17	0.0	1.1	Firmicutes	Clostridia	Clostridiales	Incertae sedis XVIII	*Symbiobacterium*	*Symbiobacterium sp.*
18	5.9	3.3	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Caloramator*	*Caloramator sp.*
19	0.0	1.1	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*Clostridium sp.*
20	0.0	5.6	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*Clostridium*
21	0.0	5.6	Proteobacteria	Beta-Proteobacteria	Burkholderiales	Alcaligenaceae	*Achromobacter*	*Achromobacter*
22	2.9	2.2	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	*Desulfotomaculum*	*Desulfotomaculum*

23	2.9	1.1						Unc. Bacterium
24	2.9	2.2	Firmicutes	Bacilli	Bacillales	Bacillaceae	*Lysinibacillus*	*Lysinibacillus fusiformis*
25	1.5	2.2	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	*Clostridium*	*Clostridium sp.*
26	2.9	0.0	Unc. Bacteroidetes					
27	1.5	0.0	Firmicutes	Clostridia	Clostridiales	Peptococcaceae	*Desulfosporosinus*	*Desulfosporosinus*
28	1.5	0.0	Proteobacteria	Epsilon-Proteobacteria	Campylobacterales	Campylobacteraceae	*Arcobacter*	*Arcobacter sp.*

The results of the ARDRA analysis showed that nearly half (46%) of the bacterial population can be assigned to the phylum Firmicutes. 30% belongs to the class of Clostridia, 15% to the class of Bacilli. The phylum Bacteroidetes and so far uncultured bacteria appeared 25% each of the whole community. The remaining 4% were analyzed as Proteobacteria (Table 4).

The most abundant bacteria in the phylum Firmicutes were *Clostridium sp*. One species was found to be *Caloramator sp*. For example, the assigned *Caloramator sp*. ferments glucose and some other sugars to acetate, isobutyrate, isovalerate, valerate, lactate, and ethanol and is also able to degrade xylan but not cellulose (Collins et al., 1994). In the order Clostridiales the species *Butyrivibrio sp.* ferments glucose to formate, butyrate, lactate, succinate and ethanol and plays an important role in the protein degradation (Kopecny et al., 2003). Further a *Sedimentibacter sp*. was found besides three members of the Peptococcaceae namely *Desulfotomaculum*, *Pelotomaculum* and *Desulfosporosinus sp*. *Desulfosporosinus* are gram-negative, rod-shaped, endospore-forming bacteria. In the presence of lactate they reduce sulfate and thiosulfate to sulfide. They are able to oxidize organic compounds to acetate which is the fermentation end-product and are capable to grow homoacetogenic (Stackebrandt et al., 2003; Robertson et al., 2004). *Desulfotomaculum* (Kuever et al., 1999) show similar characteristics like *Desulfosporosinus*. *Pelotomaculum* are gram-negative, anaerobic, sausage-shaped, endospore-forming bacteria. They can oxidize propionate and lactate in syntrophic association with hydrogenotrophic methanogens (De Bok et al., 2005; Imachi et al., 2002). A *Symbiobacterium* was also found. This is a gram-negative bacterium which lives in symbiosis with a *Bacillus* strain. The bacterium was found to have its temperature optimum between 45 and 65°C. However, it was also postulated that it

could live in symbiosis with other bacteria than *Bacillus* at lower temperatures (Ohno et al., 2000; Ueda et al., 2004).

Among Bacilli there were found three different species. *Lactobacillus leuconostoc* produces acids from glucose, xylose, cellobiose and other compounds (Farrow et al., 1989). Representatives of the genus *Bacillus* and *Lysinibacillus* are gram-positive, facultative anaerobic, endospore-forming bacteria. Two different species could be sequenced of the phylum Bacteroidetes. All other representatives were uncultured. Both species found, *Bacteroides nordii* and *Bacteroides acidifaciens*, belong to the order Bacteriales. *Bacteroides* are gram-negative; obligate anaerobic, non-spore forming, rod-shaped bacteria. The major end products of glucose, cellobiose or xylose fermentation are acetic acid and in minor amounts isovaleric acid, propionic acid, and formic acid. Both species can be distinguished via the 16S rDNA (Miyamoto and Itoh, 2000; Song et al., 2004).

The phylum of Proteobacteria was represented by a beta- and an epsilon-proteobacterium. *Achromobacter* belonging to the order of Burkholderiales (beta-Proteobacteria). They are gram-negative, aerobe and rod-shaped bacteria. But some species like *A. denitrificans* are able to reduce nitrogen oxides like nitrate or nitrite under anaerobe conditions to molecular nitrogen. Strains assimilate acetate, propionate, butyrate and valerate (Coenye et al., 2003). The epsilon-Proteobacterium was sequenced as *Arcobacter* which belongs to the order of Campylobacterales. This bacterium was already found in the SSCP analysis and appears in poultry manure.

It could be seen that different OTUs were sequenced as the same genus. This means that highly similar bacteria can appear as distinct OTUs. However, very low changes in the 16S rDNA can give a completely different band pattern. The ARDRA analysis was done additionally to the SSCP analysis. By means of this method it is

possible to determine a statistical appearance of species (OTUs) in the eubacterial and archeal community, respectively. On the SSCP gels it could be seen that most of the sequenced dominant bands belonged to *Clostridium sp*. These results indicate that Clostridia are dominant in the whole population. A similar result was obtained with the ARDRA analysis. There Clostridiales and *Clostridium sp*. were also dominant in the bacterial population. The class of Clostridia represented one third of all sequenced bacteria. Compared to SSCP, employing ARDRA analysis a higher number of different species was found. The advantage of the ARDRA analysis is the detection of less dominant species due to statistical probability. Actually with SSCP weak bands on the gel are difficult to amplify for the following sequencing hence only dominant bands could be analyzed.

ARDRA for methanogenic Archaea displayed a dominance of *Methanoculleus sp*. Results from R 6 day 1 indicated two different band patterns on the agarose gel (data not shown). Both represented the species *Methanoculleus*. The same result was achieved with the samples of day 21. This species belongs to the order of Methanomicrobiales. Their major substrate is H_2-CO_2 and formate to produce methane (Maestrojuan et al., 1990). Furthermore in previous studies (Krause et al., 2008; Schlueter et al., 2008) it could be seen that in reactors fed with primary maize silage and low amounts of chicken manure *Methanoculleus* was the dominant species of the archaeal community while Clostridiales were dominant in the eubacterial community.

Here we found the same organisms when using a defined soluble model substrate for maize silage indicating the value for mechanistic investigations of anaerobic digestion of maize silage.

3.4. Methanogenic activity measurement using fluorescent microscopy

Microscopic analyses based on factor F_{420} (Doddema and Vogels, 1978) provided a visual insight into methanogenic population. In the batch fermentations mainly irregular coccoid cells were observed (Fig 3). Maestrojuan et al. (1990) described in their study that *Methanoculleus sp.* are irregular coccoids and cells are 1 to 2 µm in diameter and occur singly or in pairs. Furthermore some cells appear to have flagella, but motility has not been observed. This is in accordance to the dominant species *Methanoculleus* detected in ARDRA analyses. Further clusters of these cells were seen which appeared larger in size in the presence of trace elements (Fig. 3 C). Moreover Fig. 3 D demonstrates a considerable increase in size and appearance of cell clusters in batch trials with an extra addition of 17 µM nickel which correlates to an increase of methane production and conversion of VFA`s in batch trials (Table 1 and Table 2). These results indicate in the presence of nickel the methanogenic bacteria grow faster and build more biomass. As described by Friedman et al., (1990) all methanogenic pathways converge to the enzymatic reduction of methyl coenzyme M to methane. This reduction is catalyzed by the Methyl-coenzyme M reductase complex, which includes a nickel containing cofactor called F_{430}.

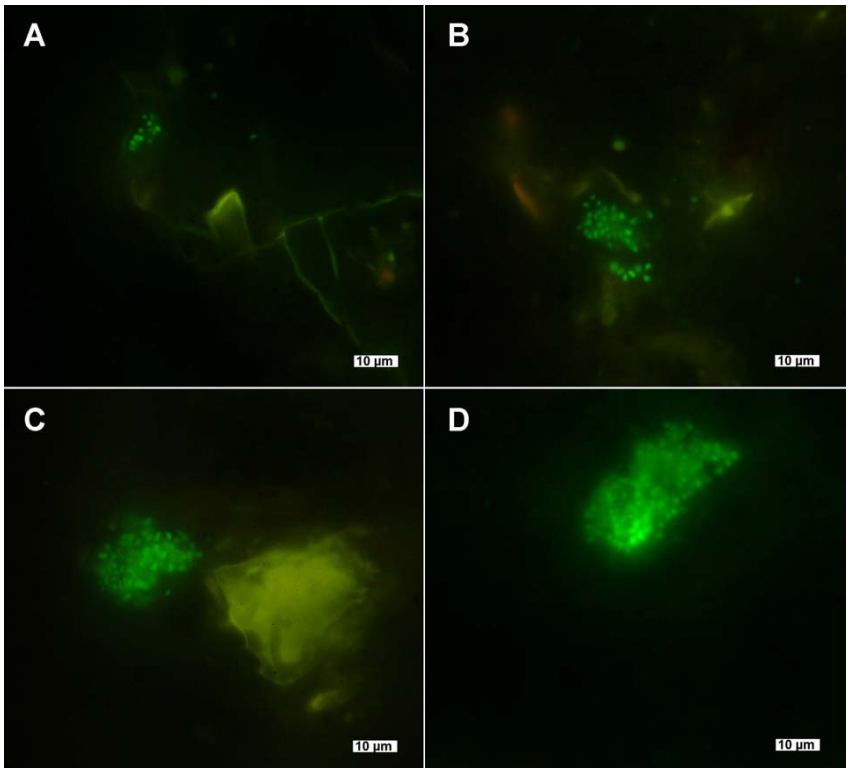

Fig. 3. Microscopic characterization of an anaerobic microbial population during degradation of maize model substrate. Fluorescence micrographs of community samples of the second batch fermentation. The samples were taken on day 21 from the blank (**A**), dS (**B**), dS+TES (**C**) and dS+TES+17 µM nickel (**D**). It can be seen that rod-shaped cells form clusters which got bigger and appeared more often with additional trace elements.

4. Conclusion

In this study an increase of biogas yields in batch fermentations upon addition of trace elements and extra nickel was demonstrated for a cellulose and starch based model substrate. Molecular biological techniques were used characterize microbial communities during anaerobic digestion of a maize model substrate. Using this tool, it was shown that the developed novel synthetic model substrate was able to mimic

maize silage since a similar microbial population was observed (e.g. *Methanoculleus* most prominent methanogen). SSCP analysis, ARDRA and fluorescent microscopy results were in agreement regarding the most prominent species found. It was also shown, that certain species such as *Arcobacter sp*. from the inoculum are significantly reduced over the duration of the batch fermentation. Future experiments should involve continuous reactors where the influence of trace elements on the microbial community should be easier to analyze first due to longer retention times and secondly due to the possibility to dilute out background concentrations of trace elements resulting from the inoculum.

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Manuscript II

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Impact of nickel and cobalt on biogas production and process stability during semi-continuous anaerobic fermentation of a model substrate for maize silage

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Abstract

The importance of nickel and cobalt on anaerobic degradation of a defined model substrate for maize was demonstrated. Five semi-continuous reactors were operated for 250 days at 35°C and a well defined trace metal solution was added to all reactors. Two reactors each were limited regarding the concentration of Ni^{2+} and Co^{2+}, respectively. At organic loading rates (OLR) above 2.6 g ODM L^{-1} d^{-1} nickel concentrations below 0.2 mg kg^{-1} FM significantly decreased biogas production by up to 25 % compared to a control reactor containing 0.8 mg Ni^{2+} kg^{-1} FM. Similarly, limitation of cobalt to 0.02 mg kg^{-1} FM decreased biogas production by about 10 %. Both limitations of nickel and cobalt lead to process instability. However, after addition of 0.6 mg nickel and 0.05 mg kg^{-1} FM cobalt the OLR was again increased to 4.3 g ODM L^{-1} d^{-1}.while process stability was recovered and a fast metabolisation of acetic and propionic acid was detected. An increase of nickel to 0.88 mg kg^{-1} FM decreased the degradation of the substrate and did not enhance biogas

performance. The increase of cobalt from 0.05 mg kg^{-1} FM up to 0.07 mg kg^{-1} FM did not influence the fermentation process.

Keywords: Biogas, Volatile fatty acids, Cellulose, Nickel, Cobalt,

1. Introduction

The reduction of greenhouse gas emission is a commonly discussed and important issue. Some of the major orginators of CO_2 emissions to the atmosphere are oil, natural gas and coal fired power plants (Gerin et al., 2007). Anaerobic digestion and production of biogas is one of the future technologies to convert biomass to energy in form of power, heat and fuel. In addition to co-fermentation of biowaste, liquid manure and agricultural biomass, the exclusive fermentation of renewable energy plants will be increasing. Different energy crops are used in biogas plants including maize silage (whole plant). Maize is an energy crop with high yields and energy productivity regarding cultivated area (Amon et al., 2006).

Biogas plants in central Europe, especially in Germany, are increasingly operated with maize silage as sole substrate. However, in many of these plants a dramatic decrease of biogas production was observed (Clemens, 2007). This phenomenon is usually seen a longer period after start up. A lack of specific nutrients could be one important reason for this effect. Carbon, nitrogen, phosphorus, sulphur and trace elements such as nickel and cobalt for example play an important role in the growth and metabolism of anaerobic microorganisms (Takashima and Speece, 1989; Goodwin et al., 1990). Various heavy metals act as cofactors of enzymes which are involved in the metabolic pathway of methane formation (Zandvoort et al, 2006). For example, all methanogenic pathways converge to the enzymatic reduction of methyl coenzyme M to methane. This reduction is catalyzed by the Methyl-coenzyme M

reductase complex, which includes a nickel containing cofactor called F_{430} (Friedman et al., 1990). The acetate converting enzyme complex carbon monoxide dehydrogenase (CODH) which consists of a nickel-iron-sulfur component and a corrinoid-iron-sulfur component was described (Ferry 1999). A further key enzyme in the methanogenic pathways is the cobalt/corrinoid containing methyl-H_4SPT: coenzyme M methyltransferase complex (Thauer, 1998).

Bioavailability of these obviously essential trace elements is significantly lower than their total content in the fermentation medium (Oleszkiewicz and Sharma, 1990). Therefore, the aim of this study was to determine the influence of nickel and cobalt on anaerobic fermentation dynamics before and after element-limited conditions in a semi-continuous reactor system. The process was monitored by measuring the conversion of volatile fatty acids (VFAs), the production of biogas and the stability in organic dry matter (ODM) concentration. For the first time, a well defined model substrate for maize silage was semi-continuously fermented to allow exact setting of Ni^{2+} and Co^{2+} concentrations in the process. This would otherwise be difficult with maize silage as substrate due to unknown bioavailability of Ni^{2+} and Co^{2+} contained therein. In addition, long term semi-continuous experiments should provide important information on the effect of Ni^{2+} and Co^{2+} on process stability besides biogas production which was not possible in batch-experiments as reported (Pobeheim, et al., 2010).

2. Methods

2.1. Inoculum and defined model substrate

Digester sludge from a full scale biogas plant (Fürstenfeld, Styria) fed with maize silage was utilised for reactor start up. The sludge was filterd to eliminate particles larger 4 milimeters. The inoculum sludge was diluted with distilled water to a concentration of 5 % dry substance (DS).

The defined model substrate (dS) was designed with respect to the composition of the maize plant. The main components of the maize plant are cellulose, starch and hemicellulose. Consequently, microcrystalline cellulose and starch from maize were used as carbon source in a mix of 50 % cellulose and 46 % starch. The basic nutrients carbon, nitrogen and phosphorus were mixed in an C:N:P ratio of 125:5:1. Urea was applied as nitrogen source. The adequate amount of phosphorus was supplied with a 0.1 M potassium phosphate buffer. Chemicals were purchased by Roth and Merck chemicals, germany.

2.2. Trace elements

Trace metals were added as trace element solution (Table1).

Table 1

Composition of added trace element solution

Metal	Compound	Conc. [µM]
Fe^{2+}	$FeCl_2 \cdot 4H_2O$	7.5
Zn^{2+}	$ZnCl_2$	0.5
Mn^{2+}	$MnCl_2 \cdot 4H_2O$	0.5
B^{3+}	H_3BO_3	0.1
Co^{2+}	$CoCl_2 \cdot 6H_2O$	0.8
Cu^{2+}	$CuCl_2 \cdot 2H_2O$	0.01
Ni^{2+}	$NiCl_2 \cdot 6H_2O$	0.1
Se^{6+}	$Na_2SeO_3 \cdot 5H_2O$	1.0
Mo^{6+}	$Na_2MoO_4 \cdot 2H_2O$	0.15
W^{6+}	$Na_2WO_4 \cdot 2H_2O$	0.1

Nickel ($NiCl_2 \cdot 6H_20$) and cobalt ($CoCl_2 \cdot 6H_20$) were also applied as single elements in concentrations as indicated below. Calcium and magnesium were blended as $CaCl_2$ and $MgCl_2$.

2.3. Reactor set up and operation

Semi-continuous experiments were conducted in 5 L glass reactors (GL 45, 355 x 186 mm). Five reactors were operated at 35°C (± 1°C) for about 250 days. The reactor content was mixed manually by shaking the fermenters two times per day. Experiments with nickel and cobalt limitation were carried out in duplicate. The reactors were equipped with a distributor cap to remove generated biogas from the fermenters and a valve for substrate feeding and sample collection. The fermenters were linked to a gas flow meter (Milligascounter®, Ritter, Germany) for countinous measurement of produced biogas. Furthermore, a 27 L gas sampling bag (Linde Gas; Graz, Austria) was connected to the gas flow meter to collect the biogas for determination of gas quality. The CH_4, CO_2, H_2S and O_2 content of the biogas was detected with the gas analyzer Visit-03 from the Messtechnik EHEIM GmbH (Schwaigern, Germany). The gas analyzer was calibrated with a mix of 50 % CH_4 and 50 % CO_2 (Linde Gas, Graz, Austria).

The reactors were initially filled with 3000 g of the inoculum sludge with a concentration of 5 % DS. Thereafter, the fermenters were flushed with nitrogen gas to obtain anaerobic conditions. All reactors were conducted semi-continuously and feed 3 times per week with a 100 g mixture of appropriate amounts of the defined model substrate and an equal amount of distilled water containing trace elements as indicated below. In additon samples were directly taken from the changed reactor sludge. After day 40 of operation, addition of trace elements was started. The

complete trace element solution and extra nickel and cobalt were added to reactor R1 while the reactors R2 and R3 were conducted with a nickel limitation and reactors R4 and R5 were limited by cobalt. The organic loading rate (OLR) was continuously increased and trace elements were specific added.

2.4. Analytical methods

All trace metal measurements were conducted by ICP-OES (inductively coupled plasma - optical emission spectrometer, iCAP 6300 Duo, Thermo Fisher Scientific Inc. Waltham, MA. USA) at the ISF Wahlstedt, Germany. Samples were first prepared with HNO_3/H_2O_2 for a following microwave pressure disintegration at 180°C.

The pH was determined with a two point calibrated (pH 4 and pH 7) WTW pH 540 GLP pH-meter. A preliminary and rapid testing of produced VFA`s was carried out with the two point titration TVA/TIC technique (Burchard et al., 2001). The pH and TVA/TIC (total volatile acids / total inorganic carbonate) were determined immediately after sample collection. Nitrogen content was measured according to the method of Kjeldahl with a Vapodest Vap 50 (Gerhardt Analytical Systems, Königswinter, Germany) data not shown.

Further from fermenter samples acetic, propionic, butyric isobutyric, isovaleric and valeric acid were measured by HPLC (High-performance liquid chromatography). Thus 1 g of mixed reactor sludge was centrifuged for 10 min at 16,000 g and the supernatant was collected. An aliquot of the supernatant was pretreated according to Carrez precipitation to remove proteins and fat components. For HPLC performance a Hewlett Packard HPLC System 1100 was used. The system was equipped with aTRANSGENOMIC, ICSep ION-300, Art Nr. ICE-99-9850 column and WAGNER

LÖFFLER, ICSep ION-300, Art Nr. CH0-0800 column respectively. As pre-column TRANSGENOMIC, GC801/C, Art Nr. ICE-99-2364 and WAGNER LÖFFLER, Interaction Replacement Cart. GC-801/C,24*4,0mm, Art Nr. CH0-0831 respectively were used. Measurements were carried out at the following operating conditions: 0.005 M H_2SO_4 as mobile phase and a flow rate of 1 ml min^{-1}, injection volume 40µL, column temperature 42°C. For reporting of chromatograms special software was used (HP chemstation).

3. Results and discussion

3.1. Effect of nickel and cobalt limitation on anaerobic fermentation of a defined model substrate

In a first stage, each two biogas reactors out of five operated with a model substrat for maize silage were limited in the concentration of nickel (R2 and R3) and cobalt (R4 and R5), respectively. On the other hand, starting on operation day 40, nickel was added to reactor R1 with increasing concentrations up to a maximum of 0.88 mg kg^{-1} fresh mass (FM). In contrast, in R2 and R3 the nickel concentrations were reduced from the initial value of 0.28 mg kg^{-1} FM to 0.06 mg kg^{-1} FM by day 133 (Table 2). During this period the cobalt concentration were adjusted from 0.03 mg kg^{-1} FM (R1) and 0.04 mg (R2 and R3), respectively, to 0.19 mg kg^{-1} FM in R1, R2 and R3. Concomitantly, the organic loading rate (OLR) of the defined model substrate was increased stepwise in all five reactors to 3 g ODM L^{-1} d^{-1} (Table 2)

Surprisingly, during this period of fermentation no significant differences in biogas production and methane yield were detected between R1 supplied with nickel and R2

and R3 which were limited in the concentration of nickel (Table 2). Moreover it seems that amounts of nickel available in R2 and R3 are sufficient for a stable fermentation of the defined model substrate up to a loading rate of 2.6 g ODM L^{-1} d^{-1} while a 5 fold higher nickel concentration in R1 even slightly decreased the biogas yield (Table 3). These results were also reflected by a comparison of the theoretical and the effective biogas yield shown in Table 3. In a study of Murray and Berg (1981) it was also described that already a nickel addition of $6 \cdot 10^{-3}$ mg L^{-1} to a biowaste digester had a significant influence on enhancement of biogas production.

A decrease in process stability and biogas production was observed after increasing the OLR to 3 g ODM L^{-1} d^{-1}. Consequently, the nickel concentration in R2 and R3 was adjusted to 0.35 mg kg^{-1} FM (Table 2) while nickel concentrations in R1 were reduced constantly from day 135 (0.83 mg kg^{-1} FM) to day 250 down to 0.6 mg kg^{-1} FM. Nevertheless, R2 showed a rapid decrease of biogas production starting about day 160 with a drop down of the biogas yield to 10 % at day 168 (Table 3). Results regarding biogas production from R3 between operation day 135 and day 250 indicate an increase in process stability due to the increase of the nickel dosage up to 0.5 mg kg^{-1} FM. Interestingly, during this increase of the nickel concentration up to 0.5 mg kg^{-1} FM the addition of the defined model substrate had to be suspended twice for several days (around day 149 and 228, Table 2). Furthermore, up to day 228 the conversion rate of the defined model substrate to biogas lies in reactor R3 in average 20 % lower compared to control reactor R1 (Table 3). However, these findings further indicate the importance of nickel addition right from the beginning of the fermentation process.

The best biogas performance with OLRs up to 4.3 g ODM L^{-1} d^{-1} was obtained with a nickel concentration of 0.6 mg kg^{-1} FM in R1 as well as R3 (Table 2 and 3). This result was in agreement with findings in a previous study where batch digestion tests

of a similar maize model substrate and nickel were conducted (Pobeheim et al., 2010).

The reactors R4 and R5 were limited by cobalt. As reported in Table 2 measurements of cobalt in R4 and R5 at day 70 and 86 lead to a cobalt concentration of 0.02 mg kg^{-1} FM. Interestingly, in comparison to reactor R1 the biogas production was not as strongly affected as expected from the results with nickel (Table 3). For instance, in a study of Jarvis et al. (1997) with grass-clover silage as substrate a strong influence of cobalt was reported with a critical total concentration of about 0.02 mg kg^{-1} FM. However, the influence of nickel especially in combination with cobalt on anaerobic fermentation and the involved microorganisms could not be investigated separately. Nevertheless, Kida et al. (2001) described in their work that both Ni^{2+} and Co^{2+} were essential for the methane-producing reactions by increases of coenzymes F_{430} and corrinoids.

Upon adjustment of the cobalt concentration to 0.05 mg kg^{-1} FM in R4 and R5 at an OLR of 2.6 g ODM L^{-1} d^{-1} (day 107), in both reactors the biogas production increased up to 81 % at R4 and even 98 % at R5 (Table 3). A further increase of cobalt up to 0.07 mg kg^{-1} FM actually did not enhance biogas production in R5 and R6 (Table 2). However, with cobalt concentrations inside 0.04 and 0.07 mg kg^{-1} FM (operation day 135 - 250) and OLR`s between 2.6 and 4.3 g ODM L^{-1} d^{-1} the biogas yields of R4 and R5 average out 75 %. In comparison at the same operation period and cobalt concentrations between 0.12 and 0.19 mg kg^{-1} FM at reactor R1 the average biogas yield lies around 77 %. In addition to R4 the low conversion rate of 60 % (Table 3) at day 250 could attribute to the nickel concentration of 0.42 mg kg^{-1} FM (Table 2).

Furthermore conversion rates of 98% (day 107) in R5 and 93 % (day 168) in R4, respectively were detected with nickel concentrations as described above of about 0.6 mg kg^{-1} FM and cobalt concentrations about 0.05 mg kg^{-1} FM (Table 2 and 3).

Table 2

Summary of nickel and cobalt concentration, organic loading rate (OLR), biogas amount and methane during anaerobic digestion of a maize model substrate in 5 continuous reactors during operation time of 250 days.

Operation (day)	1	40	42	70	86	107	133	135	149	163	168	194	228	250
R1														
Ni (mg kg^{-1} FM)	0.28	0.19	**0.59**	**0.59**	**0.69**	**0.64**	**0.88**	0.83	0.78	0.75	0.71	0.63	0.61	0.60
Co (mg kg^{-1} FM)	0.10	0.03	**0.09**	**0.10**	**0.19**	**0.17**	**0.17**	**0.19**	0.17	0.17	0.16	0.12	0.14	0.15
OLR (g ODM L^{-1} d^{-1})	0.4	1.7	1.7	1.7	1.7	2.6	3.0	3.0	3.0	2.6	2.6	3.0	3.0	4.3
biogas (NL day^{-1})	0	2.4	1.8	2.4	2.5	4.4	5.1	5.4	3.0	5.2	5.2	5.3	5.7	7.5
CH_4 (%)	0	31	35	54	48	46	49	48	51	47	52	52	51	54
R2														
Ni (mg kg^{-1} FM)	0.28	0.22	0.15	0.12	0.13	0.10	0.06	**0.14**	**0.18**	**0.18**	**0.35**			
Co (mg kg^{-1} FM)	0.10	0.04	**0.09**	**0.10**	**0.19**	0.17	0.18	0.18	0.17	0.13	0.13			
OLR (g ODM L^{-1} d^{-1})	0.4	1.7	1.7	1.7	1.7	2.6	3.0	3.0	3.0	2.6	nf			
biogas (NL day^{-1})	0	2.3	2.0	2.3	3.0	4.9	4.5	4.2	2.7	3.4	0.6			
CH_4 (%)	0	39	41	47	48	44	41	45	45	35	36			
R3														
Ni (mg kg^{-1} FM)	0.28	0.20	0.15	0.13	0.14	0.09	0.06	**0.12**	**0.31**	**0.33**	**0.34**	**0.39**	**0.51**	**0.65**
Co (mg kg^{-1} FM)	0.10	0.04	**0.09**	**0.10**	**0.19**	0.17	0.16	0.18	0.16	0.13	0.13	0.10	0.13	0.13
OLR (g ODM L^{-1} d^{-1})	0.4	1.7	1.7	1.7	1.7	2.6	3.0	3.0	nf	2.6	2.6	3.0	nf	4.3
biogas (NL day^{-1})	0	2.1	1.6	2.9	3.4	4.3	3.7	3.6	1.6	4.2	4.2	4.5	2.8	8.0
CH_4 (%)	0	42	39	47	48	44	42	30	45	46	48	55	58	55
R4														
Ni (mg kg^{-1} FM)	0.28	0.21	**0.60**	**0.55**	**0.70**	**0.63**	**0.75**	**0.78**	0.74	0.59	0.55	0.56	0.53	0.42
Co (mg kg^{-1} FM)	0.10	0.04	0.04	0.02	0.02	**0.05**	**0.06**	**0.07**	0.06	0.04	0.04	0.03	**0.06**	**0.07**
OLR (g ODM L^{-1} d^{-1})	0.4	1.7	1.7	1.7	1.7	2.6	3.0	3.0	3.0	2.6	2.6	3.0	3.0	4.3
biogas (NL day^{-1})	0	1.8	1.7	1.9	2.4	4.8	5.7	5.4	4.5	4.3	5.5	5.1	4.6	5.8
CH_4 (%)	0	35	32	46	45	42	36	45	47	47	46	51	47	46
R5														
Ni (mg kg^{-1} FM)	0.28	0.22	**0.59**	**0.56**	**0.69**	**0.61**	**0.79**	**0.82**	0.79	0.61	0.62	0.58	0.54	0.51
Co (mg kg^{-1} FM)	0.10	0.04	0.03	0.02	0.02	**0.05**	**0.06**	**0.07**	0.06	0.05	0.04	0.04	**0.07**	**0.07**
OLR (g ODM L^{-1} d^{-1})	0.4	1.7	1.7	1.7	1.7	2.6	3.0	3.0	3.0	2.6	2.6	3.0	3.0	4.3
biogas (NL day^{-1})	0	2.3	2.2	1.9	2.4	5.8	4.9	5.7	5.5	4.4	4.5	3.8	5.4	7.0
CH_4 (%)	0	33	32	44	46	43	48	50	46	48	49	51	50	51

Values in bold indicate adjustment of Ni/Co concentration. Operation of R2 stopped after day 168.
FM = fresh mass
ODM = organic dry mass
NL = norm litre

Table 3

Comparison of the theoretical biogas yield to the effective biogas yield during anaerobic digestion of a maize model substrate with different concentrations of nickel and cobalt.

operation (day)	1	40	42	70	86	107	133	135	149	163	168	194	228	250
OLR (g ODM L^{-1} d^{-1})	0.4	1.7	1.7	1.7	1.7	2.6	3.0	3.0	3.0	2.6	2.6	3.0	3.0	4.3
calculated biogas yield (NL L FM^{-1} d^{-1})	0.3	1.3	1.3	1.3	1.3	2.0	2.3	2.3	2.3	2.0	2.0	2.3	2.3	3.2
effective biogas yield														
R1														
(NL L FM^{-1} d^{-1})	0	0.8	0.6	0.8	0.8	1.5	1.7	1.8	1.0	1.7	1.7	1.8	1.9	2.5
(%)	0	63	47	63	66	75	75	79	44	88	88	78	84	77
R2														
(NL L FM^{-1} d^{-1})	0	0.8	0.7	0.8	1.0	1.6	1.5	1.4	0.9	1.1	0.2			
(%)	0	61	53	61	79	83	66	62	40	58	10			
R3														
(NL L FM^{-1} d^{-1})	0	0.7	0.5	1.0	1.1	1.4	1.2	1.2	0.5	1.4	1.4	1.5	0.9	2.7
(%)	0	55	42	76	89	73	54	53	24	71	71	66	41	82
R4														
(NL L FM^{-1} d^{-1})	0	0.6	0.6	0.6	0.8	1.6	1.9	1.8	1.5	1.4	1.8	1.7	1.5	1.9
(%)	0	47	45	50	63	81	84	79	66	73	93	75	68	60
R5														
(NL L FM^{-1} d^{-1})	0	0.8	0.7	0.6	0.8	1.9	1.6	1.9	1.8	1.5	1.5	1.3	1.8	2.3
(%)	0	61	58	50	63	98	72	84	81	75	76	56	79	72

Note: calculated biogas yield are theoretical biogas yield corresponded to ODM of substrate and was calculated according to Buswell and Boyle.

3.2. Study on process stability during anaerobic fermentation of the defined model substrate

The monitoring of the continuous digestion of maize model substrate additionally included the measurement of the pH value and TVA/TIC (total volatile acids / total inorganic carbonate). An addition of the trace element solution and extra dosage of nickel and cobalt to R1 at operation day 40 (Table 2) induced a subsequent increase of the pH (Fig. 1) and a decrease of the concentration of organic acids (Fig. 2). However, a similar but less pronounced change of these parameters after exclusive addition of trace element solution was also seen for the other reactors. Nevertheless,

up to an OLR of 1.7 g ODM L^{-1} d^{-1} no significant influence of nickel and cobalt limitation on process stability was observed (Fig. 1 and 2).

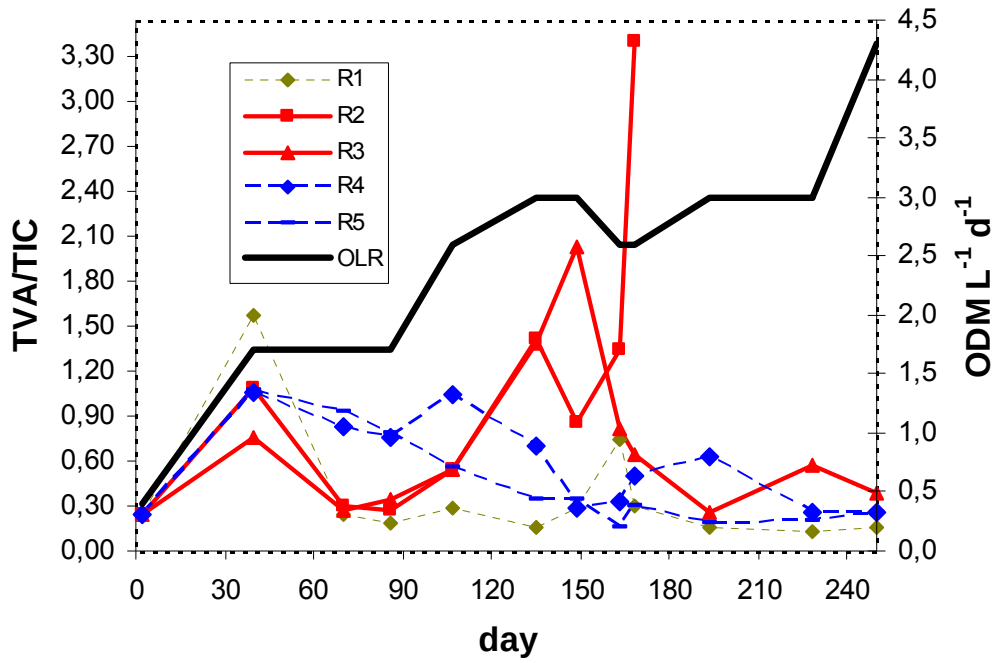


Fig. 1. pH profile and change of the organic loading rate (OLR) during anaerobic digestion of a maize model substrate with different concentrations of nickel and cobalt.

However, at a higher loading rate of 3.0 g ODM L^{-1} d^{-1} limitation of nickel as well as cobalt showed a negative impact on process stability as indicated by decreasing pH and increasing acids. This finally resulted in accumulation of organic acids with concomitant decrease of substrate conversion (Fig. 3) requiring termination of the fermentation in R2 at day 168. Reactor R3 displayed also process instability and decreased substrate degradation (Fig. 3) until operation day 228 but could be recoved by an increase of the nickel concentration to 0.51 mg kg^{-1} FM (Table 2). In contrast, in reactor R4 and R5 a continuous degradation of acids and a stable pH value above pH 7 was seen regardless increasing OLR and limitations in cobalt

content. Nevertheless, when cobalt was increased to 0.05 mg kg^{-1} FM, accumulated organic acids were metabolized immediately (Fig. 2) with a strong increase of biogas production (Table 3) and improved process stability until stop of the anaerobic fermentation after 250 days.

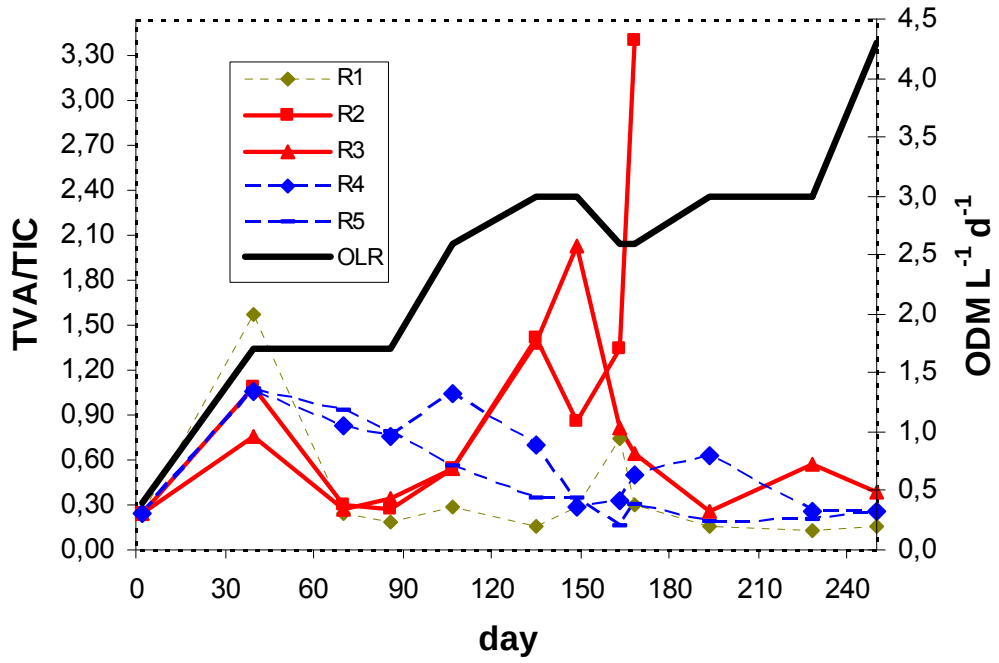


Fig. 2. TVA/TIC (total volatile acids / total inorganic carbonate) values during anaerobic digestion of a maize model substrate with different concentrations of nickel and cobalt.

The pH, TVA/TIC and ODM profiles during digestion of the maize model substrate clearly indicate that appropriate amounts of nickel and cobalt from beginning of anaerobic fermentation enhance sustainably process stability apart from conversion of the substrate into intermediates and finally biogas. In a previous study of Gonzales-Gil et al. (2003) it was reported that a continuous addition of nickel and cobalt to an anaerobic bioreactor increased bioavailability and process stability in contrast to a step by step donation of these elements.

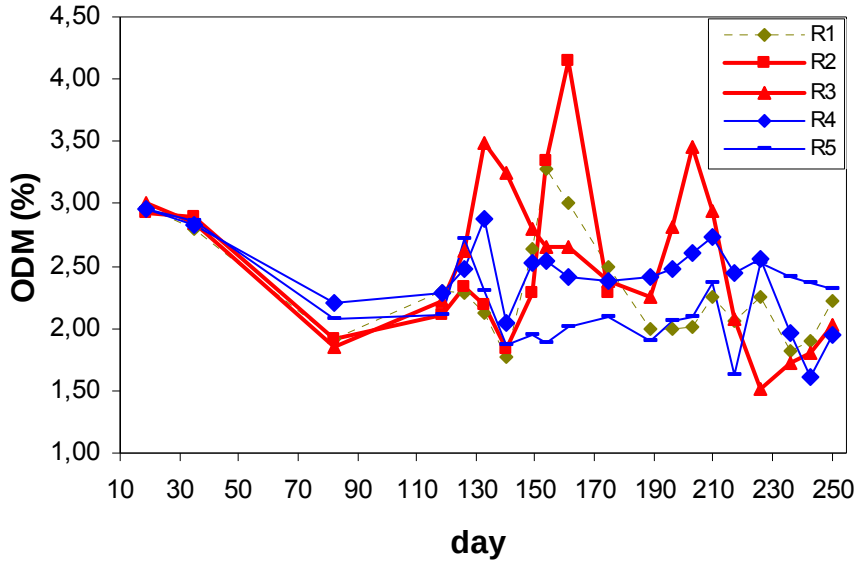
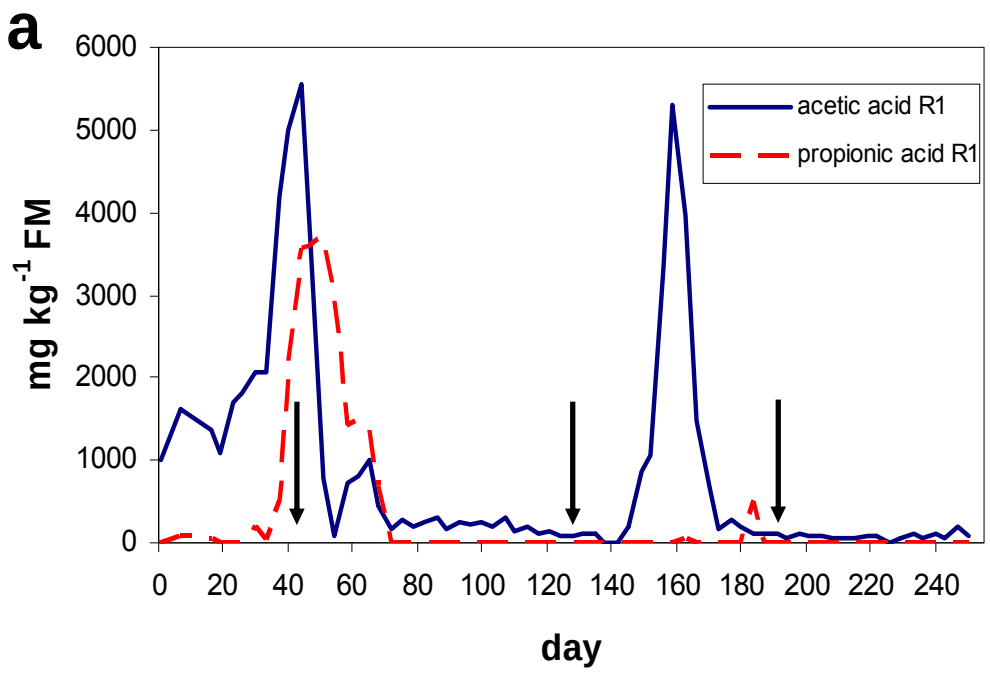


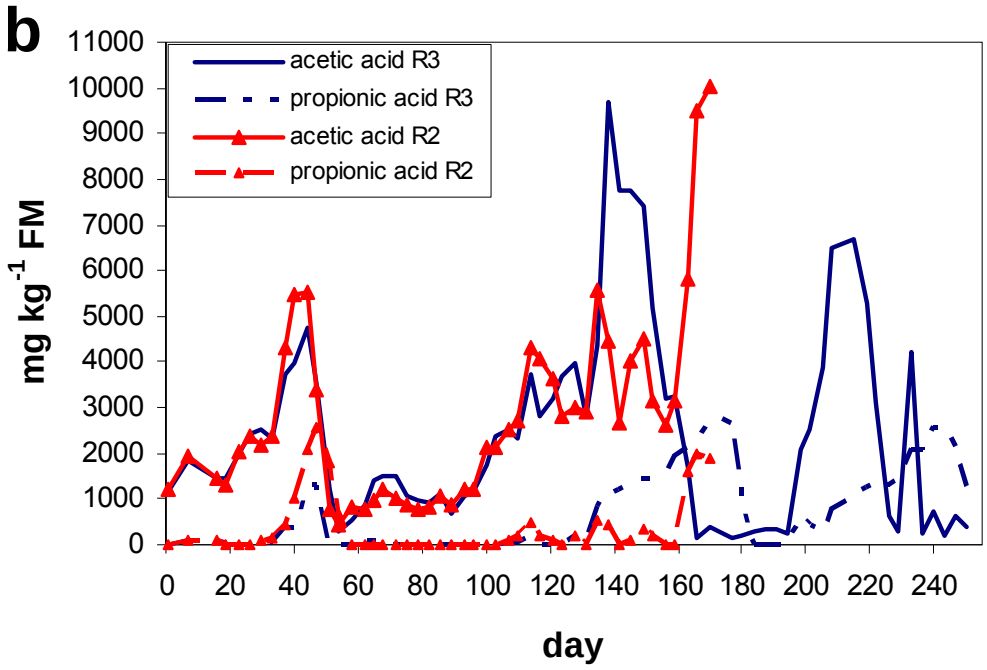
Fig. 3. Organic dry matter of R1 - R5 during anaerobic digestion of a maize model substrate with different concentrations of nickel and cobalt.

This is also reflected by the profile of acetic and propionic acid during fermentation (Fig. 4.) After addition of nickel and cobalt at day 40 to R1 in concentrations up to approximately 0.7 and 0,17 mg kg^{-1} FM acetic and propionic acid were immediately metabolized for a duration of 100 days till day 140 (Fig. 4a). However, upon adjustment of nickel to a higher level of 0.88 mg kg^{-1} FM acetic and propionic acid increased indicating no further conversion to biogas. . Consequently, readjustment to a lower concentration of 0.6 mg kg^{-1} FM supported again the conversion of these intermediates (Fig. 4a). However, in general the limitation of nickel destabilized process performance and conversion of intermediates like acetic and propionic acid as shown in Fig. 4 b. In comparison to reactor R1, accumulation of acetic acid increased up to 10^4 mg kg^{-1} FM in R2 and R3 (Fig. 4a and 4b). The limitation of cobalt in reactor R4 and R5 to 0.02 mg kg^{-1} FM between operation day 42 and 107 did also decrease the conversion of acetic and propionic acid to biogas (Fig. 4c). Furthermore, in reactor R4 an increase of propionic acid between operation day 180

and 230 upon approximately 2500 mg kg^{-1} FM with a cobalt concentration of 0.03 mg kg^{-1} FM could be detected. Nevertheless, limitation of cobalt did not show the same strong impact on anaerobic fermentation and conversion of intermediates as the limitation of nickel (Fig. 4b and 4c).

Summarizing the results of this study, the dramatic decrease of biogas production together with arising process instability seen in large scale biogas plants operated with maize silage **(**Clemens, 2007) can be amongst others attributed to limitations in nickel and cobalt. Although previous batch scale investigation indicated a negative effect of Ni/Co limitations on biogas production (Jansen et al., 2007, Murray and Berg, 1981), here, we additionally clearly demonstrate the impact on process stability. With this semi-continuous experiment it was also possible to demonstrate that all effects strongly depend on the organic loading rate. Furthermore, a stable pH and low complexation are related to bioavailability of these trace elements (Mosey et al., 1971).





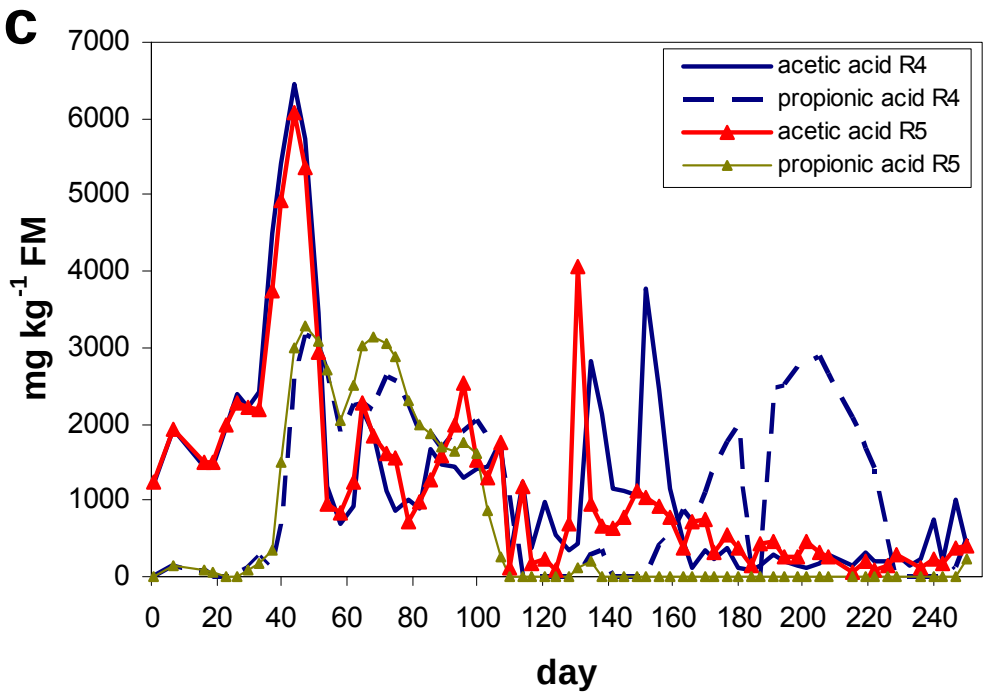


Fig. 4. Effect of variation of the organic loading rate (OLR) and trace element supplementation on the formation of acetic and propionic acid during anarobic

digestion of a maize model substrate. The first graph (a) displays results from reactor R1 with full Ni/Co dosage, black arrows indicate a change in nickel concentration. The second graph (b) shows results from reactor R2 and R3 with variation in nickel concentration and graph (c) determined data from reactor R4 and R5 with cobalt limitation (see also Table 2).

4. Conclusion

In anaerobic semi-continuous fermentations of a defined model substrate for maize, limitation of nickel as well as cobalt showed a negative impact on process stability and biogas production. Especially nickel concentrations below 0.2 mg kg^{-1} FM and cobalt concentrations below 0.02 mg kg^{-1} FM at OLR`s above 2.6 g ODM L^{-1} d^{-1} enhanced accumulation of organic acids and lead to a strong decrease of the pH-value with a concomitant decrease of methanogenic activity. On the other hand, provided that nickel and cobalt concentrations are kept around 0.6 and 0.05 mg kg^{-1} FM, stable fermentation was possible up to an OLR of 4.3 g ODM L^{-1} d^{-1}. An increase of nickel and cobalt beyond these concentrations did not further enhance biogas production.

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General conclusion

Biogas plants operating with maize silage as single substrate indicate a lack of trace elements after a couple of month which leads to a decrease of biogas production. Furthermore, this trace element deficiency results in process instability and reduced methane yields. Therefore, the aim of this study was the investigation of the influence of certain trace elements on process stability and biogas formation in anaerobic digestion of maize silage and a defined model substrate for maize.
A selected trace element solution and the single elements nickel, cobalt and molybdenum were added in different concentrations to anaerobic batch and semi-continuous experiments. Furthermore, a model substrate based on xylan (cellulose) and starch was designed to avoid addition of trace elements contained in complex substrates with unknown bioavailability.

In anaerobic batch fermentations of maize silage and the defined model substrate an increase of methane yields upon addition of the selected trace element solution as well as with nickel alone was demonstrated. Furthermore batch experiments with single cobalt also enhanced methane production compared to trials with the defined model substrate only. Batch experiments with molybdenum alone did not clearly show an enhancement of biogas production. However, batch tests with a defined model substrate for maize were verified to be a useful system for a rapid estimation of the effect of trace elements on biogas production. Nevertheless, obviously batch scale trials do generally not provide much information about potential effects on long term continuous process stability.
Therefore, in further experiments anaerobic semi-continuous fermentations of a similar model substrate as described above (cellulose instead of xylan) and limitation of cobalt and nickel were conducted. However, limitation of nickel as well as cobalt demonstrated a negative influence on process stability and biogas formation depending on the organic loading rate (OLR) of the defined model substrate. Especially the limitation of nickel at high OLR`s demonstrated a heavy increase and accumulation of volatile fatty acids and an immediately drop of the pH. Furthermore the biogas production rapidly decreased. After additional dosage of nickel to the instable fermentation process a degradation of accumulated organic acids and increase of biogas production and methane formation could be observed.

Consequently the OLR could be increased and process stability was recovered. Nevertheless, a further increase of nickel across the detected optimum did not enhance biogas production, rather a slight inhibition of biogas generation was observed. Interestingly, the limitation of cobalt did not influence process stability and biogas performance as strong as nickel limitation. This finding was also demonstrated in batch experiments and could be reconfirmed in the semi-continuous fermentations. However, the application of cobalt at an optimal concentration increases also process stability and keeps biogas production at a constant level.

Moreover, from a batch experiment with varying nickel concentrations and the defined model substrate based on cellulose eubacterial and archaeal community studies were done. Furthermore, fluorescence microscopy measurements based on cofactor F_{420} were carried out. The increased methane production with an optimal nickel concentration was also corroborated by higher activity of observed methanogenic bacteria cells. The amplified 16S rRNA genes analyzed by SSCP (single strand conformational polymorphism analysis) and ARDRA (amplified 16S rDNA restriction analysis) demonstrated that nearly the half of investigated eubacteria were identified as *Firmicutes* whereas 70% in this phylum belonged to the class of *Clostridiales* and 30% to the class of *Bacilli*. Furthermore, *Bacteroides* and uncultured bacteria represented each a quarter of the analyzed eubacterial community. Further investigations with ARDRA clearly demonstrated that the hydrogenotrophic *Methanoculleus* sp. was the dominant genus in the fermentation process which is described for maize digestion thus confirming the accordance of the defined model substrate. However, obtained results from ARDRA and fluorescent microscopy were in agreement regarding the found *Methanoculleus* sp. In addition result demonstrated that certain species such as *Arcobacter* sp. are definitely reduced over the duration of the batch fermentation. Summarizing these findings, regarding the “black box” of eubacteria and methanogenic archea in biogas plants, it was demonstrated that variation in nickel concentrations affected the anaerobic community.

The presented work demonstrates that addition of trace elements to maize related substrates improved process stability and biogas yield significantly. Moreover, both anaerobic batch experiments and semi-continuous fermentations were in agreement

regarding the concentration optimum of nickel and cobalt. Nevertheless, implementation of the obtained results in full scale biogas plants requires further investigations while the current lab scale experiments are a first indicator for the design of suitable operation conditions in anaerobic digestion of plant biomass.